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Potato Plant Gene Expression and Physiology During Three-Way Interactions with Mycorrhizal Fungi and Lepidopteran Larvae

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POTATO PLANT GENE EXPRESSION AND PHYSIOLOGY DURING THREE-WAY INTERACTIONS WITH MYCORRHIZAL FUNGI AND LEPIDOPTERAN LARVAE

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ABSTRACT

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Arbuscular mycorrhizal fungi (AMF) are integral components of ecosystems and form root associations with the majority of land plants (>80%). In these relationships, AMF provide essential nutrients to their hosts, primarily phosphorus, in exchange for photosynthates, which enhance plant growth and help plants overcome environmental stress. The below-ground interaction between plants and AMF can indirectly alter above-ground plant interactions with insect herbivores. Potatoes (*Solanum tuberosum*) which are considered one of the most important vegetable crops worldwide, naturally form symbioses with AMF. However, it is not well understood how the association between potatoes and AMF can potentially affect leaf-chewing insect herbivory. This study examined the interactions between potatoes, the generalist Lepidopteran larvae of the cabbage looper (*Trichoplusia ni*), and a generalist AM fungus (*Glomus intraradices*). The research objectives were to: a) determine the impact of the tripartite interaction involving an AM fungus, potatoes, and cabbage loopers on each organism involved, b) examine gene expression of a group of defense-related plant genes during a tripartite interaction, and c) assess changes in potato physiology during the tripartite cabbage looper-potato-AM fungus interaction. The results indicate that larval growth was negatively impacted after feeding on mycorrhizal potato plants at the low level of *G. intraradices* root

colonization (20-40% colonized at time of insect exposure) in the first experiment. Larvae gained significantly less weight after seven days of feeding on mycorrhizal plants at the low level of *G. intraradices* colonization compared to those that fed on highly colonized plants. Mycorrhizal plants at high levels of *G. intraradices* root colonization accumulated more shoot biomass, however, root biomass was not altered by the AM symbiosis. While defense-related genes were upregulated in shoots of mycorrhizal plants, their expression levels were not significantly different compared to non-mycorrhizal plants. The second and third experiments were designed using the low level of *G. intraradices* root colonization. Similarly, cabbage looper larvae gained less mass after eight days of feeding on mycorrhizal plants compared to those that fed on non-mycorrhizal plants. In this case, increased levels of transcripts of defense-related genes were detected in above-ground tissues. Interestingly, cabbage looper herbivory caused an 'apparent' stimulation of the AM fungus root colonization. Results from the third experiment revealed that while insects were negatively affected by the AM symbiosis, there were not substantial changes in potato plant physiology. Overall, this research showed that potato root colonization by *G. intraradices* indirectly altered cabbage looper growth (measured as weight), but the effect is dependent on the mycorrhizal stage. At the low level of *G. intraradices* colonization, the physiology of potato plants was not altered, but again, insects gained less mass after feeding on mycorrhizal plants. At the high levels of *G. intraradices* colonization, potato shoots accumulated more mass, but also insects gained more mass after feeding on mycorrhizal plants. Taken together, these data suggest that potatoes may transition from insect resistance to tolerance when progressing from low to high levels of G. *intraradices* root colonization.

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CHAPTER I

REVIEW OF LITERATURE

Aims

The present work was performed to evaluate the net effects of the three-way interaction among an arbuscular mycorrhizal (AM) fungus (*Glomus intraradices*) potatoes (*Solanum tuberosum*) and the generalist leaf-chewing insect, the cabbage looper (CL; *Trichoplusia ni*). Potato roots naturally form symbioses with *G. intraradices,* and plants in the field are constantly exposed to a variety of insects and pathogens. Little is known about changes in plant gene expression and physiology during three-way interactions involving AM fungus, plants and insects, therefore, gaining an understanding of the potential outcomes in these interactions could contribute to the development of environmentally-conscious methods for deterring herbivorous insect pests. Overall, the goal of this research was to develop a system for studying tripartite insect-potato-AM fungus interactions. The outcomes from this research could help in explaining the wide array of results reported in the literature and may clarify the effect the AM symbiosis has on generalist leaf-chewing insects.

Objective 1 *Determine the impact of the tripartite interaction involving AM fungi, potatoes, and cabbage loopers on each organism involved.*

H1 Cabbage looper larvae feeding on mycorrhizal potato would gain less biomass compared to those larvae feeding on non-mycorrhizal plants

H2 Shoots of mycorrhizal potato plants would accumulate more mass as a result of the AM symbiosis

H3 Damage by *T. ni* would increase transcripts of defense-related genes in *S. tuberosum*

H4 Insect-damaged mycorrhizal plants would accumulate more defense-related transcripts than insect-damaged non-mycorrhizal plants

This objective was evaluated by inoculating potato roots with varying amounts of *G. intraradices* spores to obtain distinct levels of colonization: low, medium, and high. Once the desired AM fungal density was reached, cabbage looper larvae were placed on potato leaves and were allowed to feed continuously for seven days. This experiment was performed to determine the level of *G. intraradices* root colonization that negatively affected cabbage looper fitness. Experiments in objectives two and three were designed based on the results obtained in objective one.

Objective 2 *Examine gene expression of a group of plant defense genes during tripartite cabbage looper-potato-AM fungus interactions.*

H1 AM fungus root colonization would not increase potato shoot biomass

H2 Cabbage looper larvae would gain less mass when feeding on mycorrhizal plants compared to larvae feeding on non-mycorrhizal plants

H3 Transcript levels of defense-related genes would increase in insect-damaged mycorrhizal plants relative non-mycorrhizal plants

H4 Insect herbivory would not alter AM fungus root colonization

Based on the results obtained in objective one, experiments were designed using a low number of *G. intraradices* spores. Cabbage looper fitness was evaluated by comparing larval weight gain after feeding for five and eight days on mycorrhizal and non-mycorrhizal plants. Gene expression was assessed in shoots and roots to find the possible cause for differences in larval weight gain after feeding on mycorrhizal and non-mycorrhizal plants. The impact of insect herbivory on AM fungus root colonization was quantified by staining roots.

Objective 3 *Assess changes in potato physiology during tripartite cabbage looper-potato-AM fungus interactions.*

H1 Mycorrhizal plants would experience less physiological stress responses after cabbage looper herbivory compared to non-mycorrhizal plants

H2 Cabbage looper larvae would gain less weight after feeding on mycorrhizal plants compared to larvae feeding on non-mycorrhizal plants

Mycorrhizal and non-mycorrhizal plants were exposed to five and eight days of cabbage looper herbivory. After larval removal, plant physiology measurements including photosynthesis, water potential, and relative chlorophyll abundance were taken on specific leaves. Plant fresh and dry weights and AM fungus root colonization levels were also measured.

Arbuscular Mycorrhizal Symbiosis

Arbuscular mycorrhizal fungi (AMF or AM fungi) are obligate symbionts reliant on forming relationships with plant roots and are widely distributed throughout the world. AMF are members of the Phylum Glomeromycota, and the majority of plant species (>80%) are receptive to this symbiosis (Smith and Read, 2008). This relationship has driven the coevolution of organisms belonging to these separate kingdoms for at least 450 million years (Redecker et al., 2000). Early arbuscular mycorrhizal associations with plants have been hypothesized as essential for the plant's adaptation to terrestrial environments (Schußler and Walker, 2011). AMF are generally beneficial to their plant hosts, aiding in the uptake of nutrients, mainly phosphorus (P), in exchange for carbohydrates from their host. It is estimated that at least 20% of all plant-fixed carbon is transferred to the fungus in this relationship (Parniske, 2008).

Within the plant itself, specialized fungal structures called arbuscules develop in the root cortical cells and are hypothesized to be the site for nutrient exchange (Smith and

Read, 2008). Branched extraradical hyphae represent an intermediate between plants and soils, as they mediate the uptake of elements from the soil to arbuscule-filled plant roots. Plants often grow in close proximity to one another, either as a single-species population or as members of an ecological community and can be interconnected through the hyphae of common mycorrhizal networks (CMNs) (Newman et al., 1994; Wilkinson, 1998). The web of interconnected species can transfer nutrients, including nitrogen, phosphorus, and other essential micronutrients from different individuals united by AMF (Newman et al., 1992; Meding and Zasoski, 2008). Recently, it has been demonstrated that plants linked via CMNs are able to send and receive similar signals belowground, facilitating communication between plants (Song et al., 2010).

Despite this association existing within roots, the AM symbiosis alters the entire host plant. Increased nutrient availability alters plant physiology, which leads to improved plant growth, and increased biomass accumulation in some instances (Parniske, 2008). While the increased access to nutrients confers increased fitness to cope with biotic and abiotic stresses, the additive protective features of AMF are not solely attributed to this increase in nutrients (Fritz et al., 2006). Arbuscular mycorrhizal associations positively influence soil structure, reduce greenhouse gas emissions, and improve ecosystem function and productivity by having prominent roles in nutrient cycling (Van Der Heijden et al., 1998; Bender et al., 2014; Rillig et al., 2015). Influencing plant productivity in conjunction with reducing damage caused by soil-borne pathogens has promoted the use of AMF in organic farming practices (Caron, 1989; Azcón-Aguilar and Barea, 1997; Harrier and Watson, 2004).

Role of Phytohormones in the Establishment of the Arbuscular Mycorrhizal Symbiosis

Plant roots are in constant contact with several nutrients and microorganisms in the rhizosphere. This site of water and nutrient uptake is where roots exude enzymes and phytohormones into the surrounding environment. Phytohormones, such as ethylene (ET), auxin, gibberellic acid, jasmonic acid (JA) and abscisic acid (ABA), all have roles in the plant and control virtually all aspects of development, growth, and defense (Pieterse et al., 2012). Strigolactones, a recently discovered class of phytohormones, are responsible for hyphal branching and growth of AMF towards plant roots by stimulating fungal metabolism (Besserer et al., 2006). Under phosphorus (P) or nitrogen (N)-limited conditions, plants exude larger amounts of strigolactones into the surrounding soil to trigger and promote AM symbiosis (Yoneyama et al., 2013). Conversely, under high P conditions, plants will not produce strigolactone exudates and diminish the ability to form a proper AM symbiosis (Balzergue et al., 2011). Perception of strigolactones stimulates the AMF to produce chitin oligomers. When perceived by the plant, these signals trigger calcium (Ca^{2+}) levels to rapidly fluctuate and activate the common symbiosis (SYM) pathway, which is essential for the formation of the AM symbiosis (Gutjahr et al., 2008; Genre et al., 2013). Also during this time, DELLA proteins, repressors of gibberellic acid signaling, initiate arbuscule formation (Hauvermale et al., 2012). Mutant lines with defective DELLA proteins have repeatedly shown to be incapable of forming normal functioning arbuscules (Floss et al., 2013; Foo et al., 2013). Likewise, applications of exogenous gibberellic acid prevent initial arbuscule formation (Floss et al., 2013).

Early in the establishment of the symbiosis, the AM fungus is perceived by the plant as a pathogen and must overcome the initial plant defense responses. Using effector

proteins, AMF are capable of counteracting the expression of defense-related genes. Secreted protein 7 (SP7), when secreted by the AMF *Rhizophagus irregularis*, interacts with ethylene response factor (ERF), a transcription factor, to suppress local ET signaling, normally associated with defense responses (Kloppholz et al., 2011). The phytohormones ABA and ET interact antagonistically with one another, depending on the needs of the plant (Beaudoin et al., 2000). These two hormones also have roles in maintaining functional arbuscules. Mutant tomato (*Solanum lycopersicum*) plants incapable of synthesizing ABA were poorly colonized by the AM fungus *Glomus intraradices* but became colonized once exogenous ABA was applied. In ABA-deficient roots, ET was found in elevated amounts, contributing to decreased colonization by *G. intraradices* (Herrera-Medina et al., 2007).

Arbuscular Mycorrhizal Fungi Deliver Various Nutrients to the Plant Host

Once the cortical cells of the plant root become colonized by the AM fungal symbiont, the internal components of the cell must rearrange to accommodate the forming arbuscule (Blancaflor et al., 2001). Despite being within the plant cell, the fungus retains its own cytoplasm, encapsulated by the periarbuscular membrane (a plant formed membrane). A functional AM symbiosis results in the exchange of sugars, in the form of hexoses, from the plant to the fungus, and the movement of several macronutrients from the fungus into the plant. The AM fungus utilizes thin, extraradical hyphae, capable of exploiting more of the surrounding soil to increase the nutritional status of the plant host (Allen, 2011).

The ability of AMF to directly deliver P into the root, utilizing the AM pathway versus the direct uptake pathway, is one of the primary advantages of plants forming a

symbiosis. In a well-established symbiosis, up to 100% of a plants total P can be brought in solely by the AM fungus (Smith et al., 2003). The low solubility and mobility of P within the soil creates a depletion zone surrounding the plant root, should a plant strictly utilize the direct uptake pathway (Smith et al., 2011). Because P is a growth-limiting macronutrient, P-deficient soils are indicative of low species diversity and richness (Wassen et al., 2005). In addition to contributing P to the plant, AMF acquire N by accelerating the breakdown of organic materials and transferring it to the plant host (Hodge et al., 2001; Govindarajulu et al., 2005). Transport of other nutrients, such as sulfur, by mycorrhiza has also been reported (Sieh et al., 2013). Aiding in the cycling of nutrients helps contribute to increased plant biodiversity, nutrient capture, and overall productivity of ecosystems, both above and below ground (Van Der Heijden et al., 1998). Additionally, mycorrhizae are essential components of below ground architecture and enrich soil aggregation and structure (Rillig et al., 2015). Glomalin, a glycoprotein unique to AMF, serve as aggregates for soil minerals, contributing to soil stability and erosion prevention (Rillig, 2004).

It has been shown that application of AM fungi inoculum increases yields in several agricultural crops, including members of Cucurbitaceae, Solanaceae, and Apiaceae (Ortas, 2012; Baum et al., 2015; Hijri, 2015). Enhanced nutritional status of mycorrhizal plants allows for additional resources to be allocated towards reproductive structures. Larger and more numerous flowers, as well as higher amounts of pollen, have been found in mycorrhizal plants, culminating in higher visitation rates from potential pollinators (Gange and Smith, 2005). Mycorrhizal tomato plants, inoculated with multiple species of AMF, flowered earlier in the season compared to non-mycorrhizal

counterparts (Ortas et al., 2013). These beneficial aspects of AMF contribute to enhanced reproductive success. Thus, the enhanced resistance to environmental stressors granted by AMF extends beyond the heightened nutritional status of the plant (Fritz et al., 2006).

The Role of Plant Hormones in Defense Responses

The sessile nature of plants has directed their evolution towards developing sophisticated defense systems. Chemical responses in the plant are stimulus-specific, similar to the adaptive immune system of animals (Ausubel, 2005). Plants respond to biotic or abiotic stress by altering levels of specific phytohormones. The compounds primarily involved in defense are SA, ET, JA, and ABA (Pieterse et al., 2009). In addition to regulating all developmental processes in plants, phytohormones activate defense genes against herbivores, pathogens, and competing plants (Pieterse et al., 2009; Pieterse et al., 2012). Plants must balance and allocate resources needed for continued growth and defense responses (Ludwig-Müller, 2015).

Phytohormone signaling involves increasing the expression of defense-related mRNA transcripts. In *Arabidopsis*, the defense signals SA, JA, and ET are found in varying amounts, dependent on the feeding mode utilized by the herbivores (De Vos et al., 2005). Several experiments to date have shown that inhibiting the normal functioning of these molecules can alter plant susceptibility to pathogens and insect herbivores (Hoffman et al., 1999; Wildermuth et al., 2001; Kessler et al., 2004).

It is well documented that cross-talk between hormonal signaling pathways exist. Salicylic acid is involved in the activation of systemic acquired resistance (SAR) in plants (Ryals et al., 1996). SAR-related gene activation results in broad, plant-wide changes, aimed at protecting against a variety of pathogens and can be induced by

applying exogenous SA (Ward et al., 1991). However, SA and JA are antagonistic of one another. Supplementing tomatoes (*Solanum lycopersicum*) with benzothiadiazide, an analog of SA, significantly hindered JA production, resulting in increased susceptibility and damage from leaf-chewing insects. From the same study, activating the JA pathway reduced the feeding performance of spider mites, thrips, and cabbage loopers (Thaler et al., 2002). Similarly, when confronted with *Pseudomonas syringae*, which induces salicylic-mediated defenses, *Arabidopsis* became more susceptible to pathogens normally suppressed by JA (Spoel et al., 2007).

On the contrary, ET and JA work synergistically to induce defensive responses. Challenging *Arabidopsis* mutants insensitive to ethylene (*ein1-1*) or JA (*coi1-1*) with the fungal pathogen *Alternaria brassicicola* prevented normal pathogen-induced responses, highlighting the parallel pathways between these two hormones (Penninckx et al., 1998). Additionally, *Arabidopsis* mutants deficient in linolenic acid, a precursor of JA biosynthesis, are far more susceptible to insect damage (Mcconn et al., 1997). When treated with exogenous ET, carrots (*Daucus carota*) showed elevated expression of four defense-responsive genes (*PAL, CHS, 4-CL, HGRP*) (Ecker and Davis, 1987). Ethylene response factor1, (ERF1) a downstream component of the ET pathway, shows enhanced expression immediately following applications of either ET or JA (Lorenzo et al., 2003). These cooperative compounds of interest have simultaneous involvement in plant-insect interactions and rapidly accumulate in response to wounding or herbivory (Halitschke and Baldwin, 2004; Von Dahl and Baldwin, 2007). Allene oxide synthase (*AOS*) and 12-oxophytodienoate reductase 3 (*OPR3*) transcripts, involved in JA biosynthesis, are induced in response to chewing insects (Halitschke et al., 2004; Guo et al., 2014)

Plant Secondary Metabolites in Mycorrhizal Plants

Throughout the establishment of the AM symbiosis, the fungus modulates phytohormone homeostasis in a species-specific manner (Bennett et al., 2009; López-Ráez et al., 2010; Fernández et al., 2014; Pozo et al., 2015). Once colonization is established, the AMF will actively suppress the host's SA production and will upregulate pathways associated with other hormones, namely ET, ABA, and JA (Ludwig-Müller, 2010). Despite this symbiosis occurring in roots, there are profound changes in gene expression occurring throughout the entire plant. Alterations in phytohormone levels are thought to play an important role in stress tolerance in mycorrhizal plants (Fernández et al., 2014). Transcription factors involved in the biosynthesis of secondary metabolites are regulated by phytohormone signals, as well as environmental stress (Glombitza et al., 2004; Baslam and Goicoechea, 2012; De Geyter et al., 2012). Changes in phytohormone levels and secondary metabolites brought on by the AM symbiosis have shown to enhance plant tolerance to temperature, heavy metals, drought, and salinity (Baslam and Goicoechea, 2012; Forgy, 2012; Ruiz-Lozano et al., 2012; Maya and Matsubara, 2013) when compared to NM plants. Recent RNA-seq analysis of mycorrhizal *S. lycopersicum* leaf tissue showed increased levels of transcripts of genes related to nutrient transport, photosynthesis, hormone metabolism, and abiotic and biotic stresses (Cervantes-Gámez et al., 2016). These data further support the growing body of research surrounding local and systemic resistance to biotic and abiotic stresses brought forth by the AM symbiosis in a phenomenon called priming (Conrath et al., 2006). Primed plants display stronger and more rapid defense responses, both above-ground and below-ground against

pathogens and insects. These defense responses are not constitutively expressed; instead, they are activated when needed.

Root Symbiosis with Arbuscular Mycorrhizal Fungi Alter interactions with Herbivores

The efficiency of priming in a system depends on the abundance and species of AMF and plants forming the symbiosis (Gange, 2001; Bennett et al., 2009). Studies have examined the impact of AM fungus colonization on plant interactions with other belowground organisms, including nematodes and pathogens, as they act in proximity and influence one another (Borowicz, 2001; Hol and Cook, 2005). More recently, focus has turned to investigating how AM symbioses affect interactions above-ground (Bezemer and Van Dam, 2005). Mycorrhiza-induced changes are plant-wide, affecting several aspects of plant relationships (Hause et al., 2002; Babikova et al., 2014a; Cervantes-Gámez et al., 2016). It is not surprising that the levels of the stress-related hormones, ET, JA, and SA, are greatly altered throughout the symbiosis (Fernández et al., 2014).

In an established AM symbiosis, there are significant changes in phytohormone levels (Pozo et al., 2015). Integral to the induced defense pathway and acting against microorganisms and pathogens, SA negatively affects AM fungal colonization (Medina et al., 2003), yet no consensus exists on how a developed symbiosis affects overall SA content (Fernández et al., 2014). Likewise, ET overexpressing mutants display reduced root colonization by AMF and mycorrhizal roots display lower ET levels (López-Ráez et al., 2010; Fracetto et al., 2013). Oxylipins, and JA in particular, have been found in increased levels in mycorrhizal plants (López-Ráez et al., 2010; Fernández et al., 2014). While the role of JA in AM symbioses is not well understood, the JA pathway has a well-

established role in plant defense against pests, including insects and pathogens and has been found in elevated amounts in mycorrhizal plants (Farmer and Ryan, 1990; Reinbothe et al., 2009; Wasternack, 2014; Nair et al., 2015). Modifications to phytohormone levels brought forth by AM symbioses are thought to lead to mycorrhiza-induced resistance (MIR) (Conrath et al., 2006; Jung et al., 2012).

While no consensus has been reached regarding the mechanism(s) involved in MIR, several studies have attempted to elucidate three-way interactions between insects, plants, and below-ground AMF. The outcome of the tripartite system is dependent upon each of the species involved in the system, as well as the degree of diet specialization utilized by the insect (Gange, 2007; Ali and Agrawal, 2012; Tao et al., 2016). Leaf chewing, generalist insects are generally negatively affected and gain less weight when fed mycorrhizal plants (Koricheva et al., 2009). Strawberries (*Fragaria* x *ananassa*) inoculated with either *G. mosseae* or *G. fasciculatum* of AMF showed increased resistance against black vine weevil, but the negative effect was diminished when co-inoculated with both species (Gange, 2001). Recently, beet armyworms feeding on mycorrhizal tomato (*S. lycopersicum*) gained significantly less mass compared to those fed non-mycorrhizal plants. In this instance, AMF altered plant chemistry and increased the mono- and sesquiterpines in their hosts (Shrivastava et al., 2015). Gene expression analyses further support MIR showing that insect-damaged shoots had increased transcript levels of JA biosynthesis genes and other stress-related genes (Song et al., 2013). Mycorrhizal networks connecting plants underground can receive signals from attacked plants and defensively prime themselves in anticipation of insect attackers (Song et al., 2014). Additionally, mycorrhizal plants attract parasitoids (enemies of insect

herbivores) by emitting volatile organic compounds different from non-mycorrhizal plants (Guerrieri et al., 2004).

Mycorrhizal associations are not always beneficial to the plant host. Generally, phloem feeders, such as aphids, display increased fecundity, mass, and growth when feeding on the sap of mycorrhizal plants. This could be due to the minimal damage caused by this mode of feeding as well as the increased nutritional status of the plant (Gange et al., 1999). *Mamestra brassicae* (cabbage moth), a generalist leaf-chewer, gained significantly more mass when feeding on mycorrhizal *Plantago major*, which the authors attributed to the increased nutritional and water status seen in the presence of AMF (Tomczak et al., 2016). In two species of Solanaceae, mycorrhizae were found to not have a substantial effect on the growth of *Manduca sexta* caterpillars over a 48 h feeding period (Minton et al., 2016). The most commonly used AM fungal species studied in tripartite interactions, *Glomus intraradices*, tends to have negative effects on insect feeding compared to other mycorrhizal species such as *G. mosseae* and *G. fasciculatum* (Koricheva et al., 2009). The net effects of tripartite interactions can have extensive variation within isolates of the same AMF species. Strawberry plants colonized by different AM fungal isolates of *Rhizophagus irregularis* (alternative nomenclature of *G. intraradices*), either as an individual or combined inoculum, differentially affected plant growth and insect preference (Roger et al., 2013). Therefore, generalized conclusions in these multifaceted studies should be avoided.

Significance of Studying Insect-Potato-Mycorrhizal Fungus Interactions

Over the last century, agricultural food production has significantly increased to meet the nutritional demands of over 7 billion individuals on the planet. Forests,

waterways, and all matter of natural ecosystems have undergone extensive changes to accommodate mankind's growing need for arable land. Potatoes are the leading vegetable crop in the USA and are the fourth most-consumed worldwide, with over 20 million tons produced in 2014 (Food Agriculture Organization, 2014). Potatoes are grown in a wide variety of climates and are an inexpensive source of calories. Compared to many other crops, potatoes are hardy and have much longer shelf lives. Most potatoes are processed before being exported as potato chips, dehydrated flakes, and french fries. In 2009, over 1.3 million tons of french fries were exported from the US, yielding $635 million (Economic Research Service, 2016). Further, with the recent sequencing of the potato genome, it is imperative that we understand how we can further modulate and modify its molecular mechanisms to increase agricultural outputs (Consortium, 2011).

The development of insecticides and fertilizers for agricultural use has been instrumental in providing sufficient nutrients for individuals across the world. The USA alone has tripled its consumption of many agricultural macronutrients over the last half-century, from 6.3 thousand tons in 1960, to over 19 thousand in 2011 (Ers, 2016). Increased use and demand of fertilizers has coincided with dramatic, rising prices. Between 2003 and 2013, the dollar per short ton of superphosphate ($Ca(H_2PO_4)_2$) and diammonium phosphate ($(NH_4)_2HPO_4$) fertilizer increased by over 150%. Often used alongside fertilizers, chemically-based insecticides have been used to manage insects for nearly a century. Following WWII, synthetic insecticides became widely available for use, with peak usage in the 1970's (Aspelin, 2003). Use has steadily declined due to the increased efficacy of insecticides and development of genetically modified crops, such as Bt corn. However, these methods for controlling insect populations has led to the

evolution of populations resistant to both of these treatments, further promoting insecticidal treatments (Szendrei et al., 2012; Tabashnik et al., 2013). Though both fertilizers and insecticides are essential for modern agriculture, they are prone to contaminating the surrounding environment and have far-reaching effects on multiple trophic levels within ecosystems (Savci, 2012). It is imperative that alternatives to fertilizers and pesticides be examined to potentially reduce dependency on these compounds that negatively impact the environment.

Investigating the three-way relationship between *G. intraradices*, potatoes, and CL will contribute to the growing body of knowledge of tripartite interactions. The naturally-occurring symbiosis between AMF and potatoes could be studied and managed to deter leaf-chewing insect herbivory while simultaneously increasing nutrient uptake, utilizing an environmentally-sound approach (Fester and Sawers, 2011). Insects are contributors to crop loss, either by directly feeding or by spreading pathogen-causing diseases. Therefore, it is crucial we begin to understand the mechanisms involved in MIR to meet the agricultural and environmental demands of the future. This research focused on developing a robust system to study insect-crop-AM fungus interactions to better understand how the AM symbiosis indirectly alters a chewing insect feeding above-ground.

CHAPTER II

THE EXTENT OF ARBUSCULAR MYCORRHIZAL FUNGUS ROOT COLONIZATION HAS VARYING EFFECTS ON INSECTS FEEDING ABOVE-GROUND

Abstract

Arbuscular mycorrhizal fungi (AMF) are integral components to ecosystems, contributing to nutrient cycling, plant growth, and fitness. Plants often interact simultaneously with AMF below-ground and with insect herbivores above-ground. AM symbiosis can grant protective effects against abiotic stresses, and more recently, mycorrhiza-induced resistance against insects has been proposed. However, few studies have examined how the progression of AM fungus root colonization affects insect herbivores above-ground. This study examined whether the extent of colonization by the AM fungus *Glomus intraradices* affected potato plants during herbivory by a generalist caterpillar, the cabbage looper (*Trichoplusia ni*). This was accomplished by inoculating potato roots with varied amounts of *G. intraradices* spores to obtain low, medium, and high levels of colonization, followed by exposure to seven days of continuous insect feeding in shoots. Cabbage loopers that fed on highly-colonized plants gained significantly more biomass compared to those that fed on less-colonized plants. However, no differences in insect mass were detected between any of the mycorrhizal treatments and control. Further, highly-colonized plants gained significantly more shoot mass, but roots were unaffected in all treatments. While defense-related genes were modestly

induced in shoots of insect-damaged mycorrhizal plants, transcript levels were relatively similar in all plants. The results of this experiment did not support our hypotheses that mycorrhiza-induced resistance occurred at every level of *G. intraradices* root colonization, however, the data suggest that larvae may be negatively affected by low levels of AM fungus colonization.

Introduction

In undisturbed areas and agricultural environments, plants are exposed to detrimental biotic and abiotic stresses, such as insect herbivory and soil nutrient limitations. To help alleviate nutrient-related stresses, nearly three-quarters of flowering plants interact with members of the fungal phylum Glomeromycota to form symbiosis between roots and arbuscular mycorrhizal fungi (AMF or AM fungus) (Smith and Read, 2008; Brundrett, 2009). AMF are obligate symbionts that aid in the uptake of nutrients, primarily phosphate (P), in exchange for carbohydrates from the plant host (Smith et al., 2011). In P-deficient soils, plant roots will readily form symbioses with AMF to meet nutritional needs (Liu et al., 2007). Conversely, P-rich soils hinder both quality and function of AM fungal species, preventing normal formation of the AM symbiosis (Gosling et al., 2006; Balzergue et al., 2011).

Enhanced nutritional availability to the plant host confers improved growth and overall fitness in comparison to non-mycorrhizal plants (Goverde et al., 2000; Gange and Smith, 2005; Parniske, 2008). In addition to improved nutrition, some mycorrhizal plants have shown enhanced resistance to biotic and abiotic stresses (Fritz et al., 2006; Koricheva et al., 2009; Song et al., 2013; Shrivastava et al., 2015). The mechanisms of heightened defense, though not fully understood, are thought to be accomplished by

modulation of phytohormone levels. Throughout the relationship with the AM fungus, phytohormone levels fluctuate, with some hormones increasing while others decrease (Medina et al., 2003; Herrera-Medina et al., 2007; Pozo et al., 2015). Abscisic acid (ABA), salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) have roles in maintaining plant homeostasis, growth, defense (Pieterse et al., 2012) and control the development and maintain the AM symbiosis (Gutjahr, 2014). In addition, hormone biosynthesis is affected by the mycorrhizal status of the plant (Walker et al., 2012). JA is of notable interest due to its integral role in generating insect-deterrent compounds (Baldwin, 1998). Production of JA is correlated to major defensive responses of plants, including protease inhibitors (Moore et al., 2003; Chen et al., 2005), and volatile organic compound (VOC) production, (Farmer and Ryan, 1990; Schmelz et al., 2003) with the intent of attracting herbivore parasitoids and 'warning' nearby plants of incoming attacks (Arimura et al., 2000). Neighboring plants become 'primed' from these airborne signals, displaying stronger and more rapid cellular defense responses to biotic stress (Engelberth et al., 2004).

Defensive 'priming', in addition to being triggered by VOCs, can be stimulated by below-ground symbiosis with AMF in a phenomenon called mycorrhiza-induced resistance (MIR) (Pozo and Azcón-Aguilar, 2007; Song et al., 2013). In a well-established symbiosis, local and systemic changes take place, where increased transcription of defense-related genes (Liu et al., 2007; López-Ráez et al., 2010) and subsequent insect antifeedant compounds accumulate (Vannette and Hunter, 2009; Shrivastava et al., 2015). Past studies focusing on MIR have shown contradictory results. This could be attributed to differences in feeding mechanisms utilized by insects

(Koricheva et al., 2009) and the species-specific interplay between plants and AMF (Bennett et al., 2009; Vannette and Hunter, 2013). The degree of diet specialization also contributes to the effectiveness of MIR, with generalist, chewing insects showing sensitivity to mycorrhiza (Koricheva et al., 2009; Ali and Agrawal, 2012). Chewing insects accumulated less biomass after feeding on mycorrhizal plants (Gange et al., 2002; Shrivastava et al., 2015). Plant defense-related genes were induced in mycorrhizal plants damaged by caterpillars (Song et al., 2013). In some instances, insect performance is enhanced by AMF colonization (Goverde et al., 2000), though this is generally seen in phloem-feeding insects (Gange et al., 1999; Koricheva et al., 2009).

Previous studies have focused on AM fungal species and diversity in insect-plant-AM fungal interactions (Gange, 2001; Klironomos, 2003; Bennett and Bever, 2009; Bennett et al., 2009; Roger et al., 2013). However, few studies have examined the effect of AM fungi at multiple levels of root length colonization (Garrido et al., 2010; Vannette and Hunter, 2013; Tao et al., 2016). A prevailing hypothesis states that the degree of benefit changes over the course of the AM symbiosis, meaning that as plant roots become more colonized, the benefits to the plant will change, described as a curvilinear model (Gange and Ayres, 1999). The proposed model depicts mycorrhizal colonization as having an ‘optimal’ level, where P uptake through mycorrhiza contributes to enhanced growth of the host. However, once ‘optimal’ colonization is exceeded, the increased demand of carbon from the host outweighs the benefits provided by the symbiosis. Further, high AM fungal density could hinder plant growth by competing for soil nutrients, affecting native soil microflora. This same phenomenon was supported by Garrido et al. (2010), where characteristics including seed production, foliar area, and

root mass of mycorrhizal *Datura stramonium* plants increased significantly, reached a plateau, and then decreased, proportional to the extent of root colonization. Following artificial defoliation, plant tolerance was found to decrease linearly with AM fungal density. It was noted that at low density of AM fungus colonization, plants had the capacity to allocate more resources towards overcoming shoot defoliation, yet the degree of defoliation was mechanical without insect elicitors.

Here, we investigated the effects of three levels of AM fungal root density on the polyphagous insect, the cabbage looper (*Tricoplusia* ni) after feeding on potatoes (*Solanum tuberosum,* 'Desirée'). Our primary objective was to determine the impact of the tripartite interaction between potatoes, cabbage loopers and the AMF *G. intraradices*. We hypothesized that 1) cabbage loopers feeding on mycorrhizal plants would gain less biomass; 2) shoots of mycorrhizal plants would accumulate more mass; 3) insect-damaged mycorrhizal plants would exhibit increased transcription of genes involved in plant defenses; 4) mycorrhizal plants would accumulate more defense-related transcripts than non-mycorrhizal plants.

Materials and Methods

Plant Growth and Arbuscular Mycorrhizal Fungus Inoculation

Potato (*Solanum tuberosum*, 'Désirée') nodal clones were propagated *in vitro* using Murashige and Skoog medium, supplemented with 20 $g.L^{-1}$ sucrose and 4 $g\ L^{-1}$ phytagel at a pH of 5.7 (Murashige and Skoog, 1962). Plantlets were grown for seven weeks (22°C, 16 h photoperiod) until adequate root systems developed. *Glomus intraradices* spores provided by Dr. Maria Harrison (Boyce Thompson Institute for Plant Research) were maintained on carrot roots in bi-plates (St-Arnaud et al., 1996). Multiple

potato cultivars, including Désirée, are known to naturally form symbiosis with *G. intraradices* (Rausch et al., 2001; Senés-Guerrero et al., 2014). Plants were transplanted to 12 cm x 9 cm pots containing 250 mL of 9:1 mason sand: topsoil (Pioneer Sand Company, Windsor, CO, USA) substrate. Previous work done by our lab showed a 9:1 ratio to be the most efficient at promoting the symbiosis. Sand was washed multiple times using deionized water before being autoclaved (121°C, 20 PSI, 60 min). The topsoil was passed through two sieves (USA Standard sieve no. 16 and 8) to remove any large particulates before autoclaving three times (121°C, 20 PSI, 60 min). Nutrient analysis of the substrate was conducted at Weld Laboratories in Greeley, CO (Table 1). Field-grown potatoes require approximately 13.6 kg phosphorus per hecacre for sufficient growth (http://www.extension.uidaho.edu/nutrient/crop_nutrient/potato.html), which far exceeded the 6.4 kg per acre found in our soil substrate (Table 1). Newly transplanted potatoes were kept under plastic domes to maintain high humidity. Plants were grown in laboratory conditions under four fluorescent bulbs (113-148 µmol $m^{-2}s^{-1}$ lighting) with a 16-hour light photoperiod.

Table 1. Macronutrient analysis of soil substrate (9:1, sand: topsoil) used in experiments before and after autoclaving.

	Pre-autoclaved		Autoclaved	
pH	7.47		7.18	
Organic matter (%)	0.20		0.10	
	ppm	kg/hectare	ppm	kg/hectare
Nitrate	3.5	5.8	3.4	5.9
Phosphorus	11.0	18.1	4.0	6.4
Potassium	22.0	35.8	20.0	31.8

G. intraradices spores were retrieved from plates using 10 mM sodium citrate (pH 7.9) to dissolve the media. Spores were suspended in autoclaved MilliQ® water. After one week of acclimation, plants were transplanted to new pots containing 250 mL of sterile 9:1 sand: topsoil substrate. Potato roots were inoculated using a micropipette with a solution containing 250, 500, and 800 *G. intraradices* spores per plant, designated as low, medium, and high spore density, respectively. Our target colonization before placing insects was 20-40% for low, 40-60% for medium, and >60% for high. Non-mycorrhizal plants received a 'mock' filtered solution obtained from the final rinse of the spore preparation. Mock- and *G. intraradices*-inoculated roots were covered with a top layer of the sand-topsoil substrate from the original pots (~250 mL) containing root exudates. Potatoes were grown under laboratory conditions (20-23°C, 16 h photoperiod) and were fertilized twice per week with 35 ml of half-strength modified Hoagland's solution with reduced P (100 µM P) to promote AM fungus colonization (Liu et al., 2007).

Extra potted plants inoculated with the same number of spores as the experimental plants were used to evaluate the progression of *G. intraradices* colonization. A random subsample of potato roots were cleared in 10% (w/v) KOH for 6 h at 85°C, followed by rinsing in deionized water, and staining with 5% (v/v) Schaeffer black ink prepared in 5% (v/v) acetic acid (Vierheilig and Piché, 1998). *G. Intraradices* root colonization was quantified using the gridline-intersect method (Mcgonigle et al., 1990). Test pots indicated the colonization to be at 11% for low, 19% for medium, and 30% for high mycorrhizal plants at 31 days after inoculation. At 41 days post-inoculation, plants were moved to the greenhouse (19-24°C day, 17-23°C night, 16 h photoperiod) in BugDorm 2

insect rearing tents, (BioQuip Products, Rancho Dominguez, CA, USA) separated by treatment group. Tents containing the plants were rotated clockwise throughout the greenhouse during the experiment to ensure all plants received similar light, humidity and temperature conditions.

Insect Herbivory and Tissue Collection

After plants acclimated to the greenhouse for eight days, second-instar cabbage loopers (*Tricoplusia ni*) purchased from Frontier Agricultural Sciences (Newark, DE, USA) were organized into groups of five and weighed prior to placement on the uppermost expanded leaf of each plant. Once mycorrhizal plants reached target colonization levels (low, medium, and high), 'insect-damaged plants' were continuously exposed to cabbage looper herbivory for seven days. Prior to harvesting, *T. ni* larvae were removed and weighed using a microbalance (MX5, Mettler Toledo, Columbus, OH). Shoots and roots were collected and weighed separately to obtain fresh weights. A random root subsample was taken from each plant for staining of *G. intraradices*. Plant tissue was frozen in liquid nitrogen before storing at -80°C. Two additional plants from each treatment were harvested and weighed before being dried at 85°C for 48 h (Babikova et al., 2014b). Dried shoots and roots were weighed to obtain fresh weight: dry weight conversion ratios for all plants and treatment groups.

Ribonucleic Acid (RNA) Isolation and Complimentary Deoxyribonucleic Acid (cDNA) Synthesis

Shoot and root tissues were ground in liquid nitrogen to a fine powder using a mortar and pestle. All materials were decontaminated before use with RNase AWAY (Molecular BioProducts, San Diego, CA, USA). Total RNA was isolated from 50-100 mg

of shoot tissue using 1 mL of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) per manufacturers instruction with minor modifications. To reduce polysaccharide contamination, 33.3 ul of 10% (w/v) polyvinylpyrrolidone (wt 40 000) was added following the TRIzol® step. RNA samples were treated with 3 µl Turbo™ DNase (Ambion, Foster City, CA, USA) and 10 µl of 10X reaction buffer to a total volume of 87 µl. Samples were incubated at 37°C for 40 min. RNA was purified using chloroform and subsequent isopropanol precipitation. For cDNA synthesis, one µg of total RNA resuspended in 11 µl of RNAse-free water was mixed with 1 µl each of dNTPs (10 mM) and anchored oligo dT_{22} (500 ng μl^{-1}). The final volume of 13 µl was incubated in a T100™ thermal cycler (Bio-Rad) for 5 min at 65°C, and placed on ice. Following, 4 µl of SSIV buffer, 1 µl of DTT (100 mM), 0.3 µl SuperScript® IV (Invitrogen), 1.2 µl DEPC-treated water, and 0.5 µl RNase OUT were added to obtain 20 µl reactions. Samples were incubated at 50°C for 10 min followed by 80°C for another 10 min.

Primer Design and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Potato sequences were obtained via the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) and SPUD database *(*potato.plantbiology.msu.edu/). Query sequences either from tomato, tobacco, *Arabidopsis*, or *Medicago truncatula* (based on gene availability) were used for BLAST searches. Once the correct potato sequences were confirmed, oligonucleotides were designed using primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) or primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). A number of reference genes obtained from the literature were tested, but oligonucleotides were re-designed for qPCR.

Two reference genes (elongation factor 1α and β tubulin) showed consistent results using shoot and root samples. Oligonucleotide sequences for target genes and reference genes are listed in Table 2. Each qPCR reaction consisted of 1 µl of cDNA template (1:3), 2 µl autoclaved MilliQ® water, 5 µl of Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µl (3 µM each) of forward and reverse primers. Each 384-well plate was run on a C1000® Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The thermal profile consisted of an incubation for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and annealing/extension for 1 min for 55-60°C, ending with melt curve analysis (65-95°C incrementally increasing by 5°C). The relative expression of target genes was calculated using the $2^{-\Delta Cq}$ method (Schmittgen and Livak, 2008). Each target gene was normalized to the geometric mean of both reference genes (ΔCq).

Table 2. Oligonucleotide sequences for potato genes

Gene	Sequence (5' to 3')	Amplicon length (bp)	Plant species used to find potato sequence	Potato Sequence ID
Elongation factor 1 α (*EF1-α*)	F: GAGACCTTTGCTGAATACCCAC R: TCACTTTGGCACCAGTTGG	118	Potato	Sotub06g010760.1.1
β-tubulin (β*tub*)	F: ACCAGGATGCTACAGGAGATG R: GGCAGAAATTGAACAAACCAA	119	Potato	PGSC0003DMT400025739
Allene oxide synthase 1 (*AOS1*)	F: AATGGGTCGGAAACAGAGAACC R: GGAGGAACAATTCGACCAACAA	104	Tomato	PGSC0003DMT400043495
12-Oxophytodienoate reducatase 3 (*OPR3*)	F: TCACAAAGGAGCAAGTAGAGGA R: GAGATGCACGACCAACATGCC	103	Tomato	PGSC0003DMT400079356
9-cis-epoxycarotenoid dioxygenase (*NCED*)	F: GCCGTTCAATTCAAAAATGG R: ATTCACCAATGGCTTTAGGG	115	Tomato	PGSC0003DMT400071048

Statistical Analyses

Raw data were tested for normality using the Shapiro-Wilk test. Shoot weight, root weight, AMF colonization, and relative gene expression was subjected to analyses of variance (ANOVA), with subsequent post-hoc comparisons using Tukey's honestly significant difference (HSD) test ($P<0.05$). Student's *t*-tests were performed for CL weight data. All statistical analyses were conducted using SAS (9.4 for Windows) software.

Results

Shoot Growth Increases When Potato PlantsExhibit High Levels of *Glomus intraradices* Root Colonization

Shoots and roots were collected separately and weighed before being frozen in liquid nitrogen for further gene expression analysis. Consistent with previous research (Goverde et al., 2000; Parniske, 2008), mycorrhizal plants gained more above-ground mass compared to NM plants grown under the same conditions (Fig. 1A). Highly colonized plants were substantially larger than NM plants. Additional differences were found in shoot biomass in low and medium levels of colonization, but were not significant. There was no variation in the fresh weights of collected roots. To account for potential differences in water, two replicates from each treatment were weighed after drying for 48 h. Fresh weight: dry weight conversion was applied to both shoots and roots to generate dry weights for all shoots and roots. Highly colonized plants had significantly more biomass against all other treatment groups (Fig. 1B). No differences were found between the low, medium and control plant shoots. Dry weight of roots in all plants were similar.

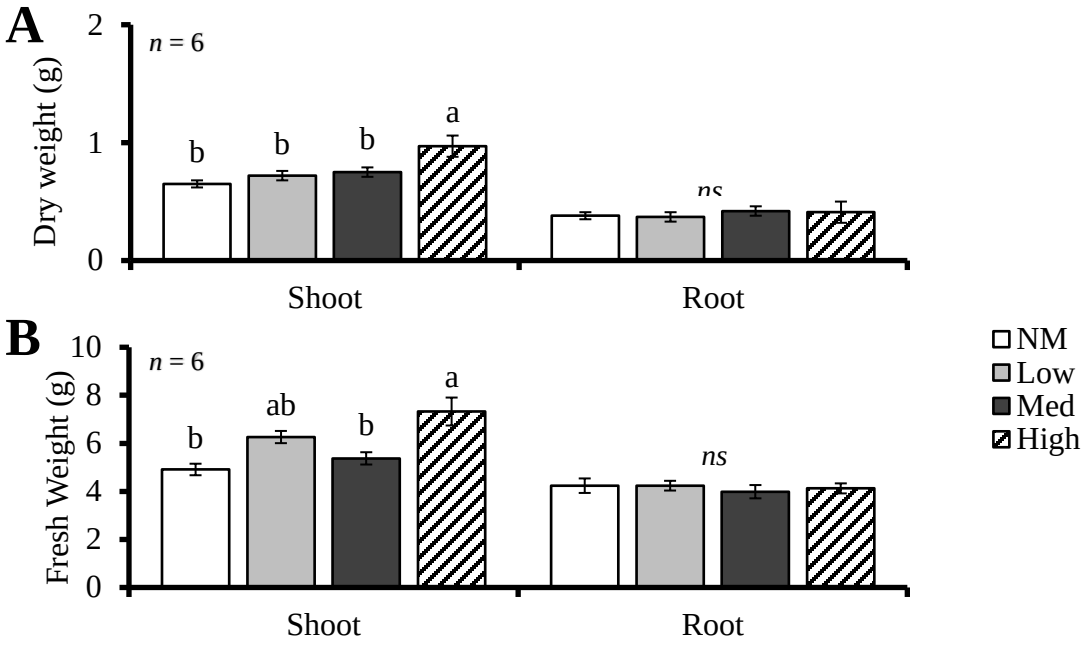

Figure 1. Effect of the extent of *Glomus intraradices* colonization and cabbage looper (*Trichoplusia ni*) herbivory on potato (*Solanum tuberosum)* growth. Once mycorrhizal plants reached target *G. intraradices* root colonization levels (low, medium, and high), insects were added to plants and fed for seven days. **(A)** Fresh weight of shoots and roots, **(B)** Dry weight of shoots and roots. Tissues were dried for 48 h at 85°C to obtain dry weights. Values represent mean ± standard error. Biological replicates for each treatment indicated along y-axis. Different letters indicate significant differences based on Tukey's HSD test ($P < 0.05$) for that tissue type. NM = non-mycorrhizal plants

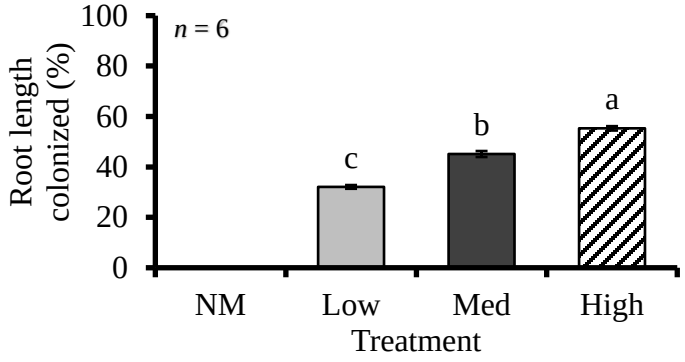

Figure 2. Potato root colonization by *Glomus intraradices* after seven days of cabbage looper (*Trichoplusia ni)* herbivory. Potato roots were inoculated with approximately 250, 500, 800 *G. intraradices* spores to obtain low, medium, and high levels of colonization, respectively. At 41 days post-inoculation, plants were exposed to seven days of cabbage looper feeding. Root colonization was quantified after insects were removed. Values represent mean ± standard error. Biological replicates for each treatment indicated along y-axis. Different letters indicate significant differences based on Tukey's HSD test ($P < 0.05$). NM = non-mycorrhizal plants.

Inoculating Potato Roots with Varied Amounts of *Glomus intraradices* Spores Leads to Distinct Levels of Arbuscular Mycorrhizal Fungus Colonization

As was expected, the results showed that inoculating potato roots with varying amounts of *G. intraradices* spores affects the advancement of the below-ground colonization (Fig. 2). We achieved three distinct mycorrhizal treatments in addition to 'mock' inoculated plants that showed no colonization.

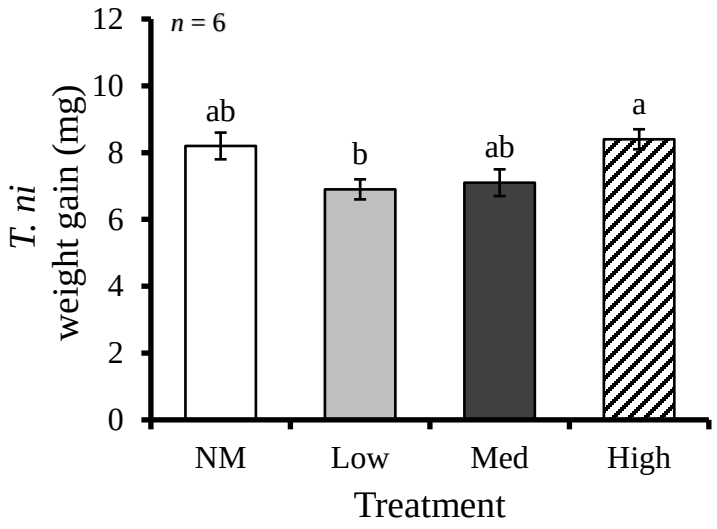

Figure 3. Indirect effect of the extent of *Glomus intraradices* colonization of potato (*Solanum tuberosum)* on cabbage looper's (*Trichoplusia ni*) larval growth. Once mycorrhizal plants reached target *G. intraradices* colonization levels (low, medium, and high), insects were added to plants. Mean weight gained by individual larva after feeding on potato shoots for seven days. Biological replicates for each treatment indicated along y-axis. Values represent mean ± standard error. Different letters indicate significant differences based on Tukey's HSD test ($P < 0.05$). NM = non-mycorrhizal plants.

Cabbage Looper Larvae Gain Less Weight After Feeding for Seven Days on Mycorrhizal Plants at Low Levels of *Glomus intraradices* Root Colonization

The goal of this experiment was to determine whether the level of *G. intraradices* root colonization had an indirect impact on cabbage looper larval growth after feeding for

seven days on mycorrhizal plants versus non-mycorrhizal plants. Our data indicate that larvae gained less weight after feeding on mycorrhizal plants that exhibited low levels of *G. intraradices* root colonization compared to larvae that fed on plants at high levels of colonization (Fig. 3). Given the relatively short duration of feeding in this experiment, we did not find statistical differences between larvae that fed on non-mycorrhizal plants relative to those that fed on mycorrhizal plants (Fig. 3).

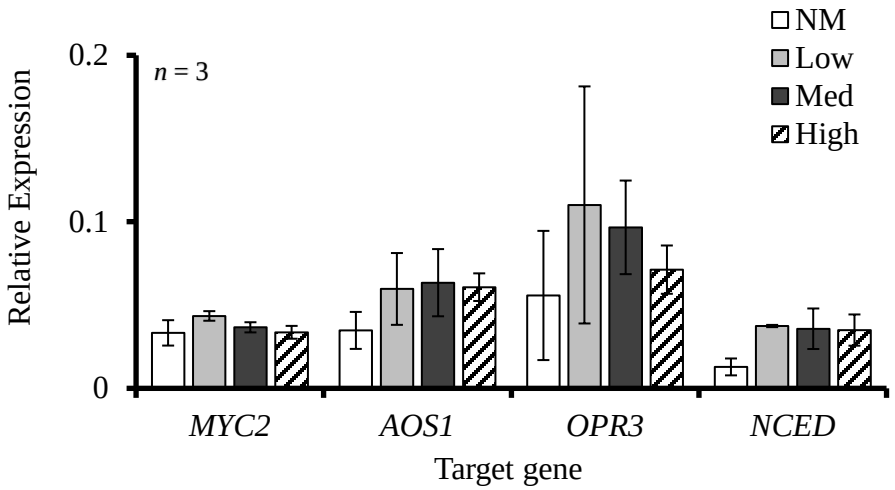

Figure 4. Relative gene expression in mycorrhizal and non-mycorrhizal potato (*Solanum tuberosum*) shoots after seven days of cabbage looper (*Trichoplusia ni*) herbivory. Potato plants were inoculated with varied amounts of *Glomus intraradices* spores to achieve three distinct levels of colonization (low, medium, high). Values present the mean of three biological and two technical replicates per treatment. Relative expression was generated by normalizing each target with the geometric means of elongation factor 1 alpha and β-tubulin, and by using the formula $2^{-\Delta Cq}$. Statistical significance among treatments for each gene was determined using one-way ANOVA ($P<0.05$). NM = non-mycorrhizal plants.

Subtle Changes in Gene Expression Occur in Mycorrhizal Plants After Seven Days of Cabbage Looper Herbivory

To investigate changes in plant gene expression during tripartite interactions, a handful of genes known to be involved in JA biosynthesis were chosen (López-Ráez et al., 2010). Allene oxide synthase 1 (*AOS1*) and 12-oxophytodienoate reductase 3 (*OPR3*),

two essential protein-coding genes in the 13-lypooxygenase (LOX) branch of the JA biosynthesis pathway were found to be induced after cabbage looper herbivory. However, their expression was not significantly different between any mycorrhizal groups (Fig. 4). Transcription factor *MYC2* expression is induced by JA, promoting wound-responses to herbivory (Lorenzo et al., 2004). Similarly, no differences were found in *MYC2* expression. 9-cis-epoxycarotenoid dioxygenase (NCED) is a key enzyme in ABA biosynthesis and was not found to be altered in any of the mycorrhizal treatments.

Discussion

Plants serve as links between above- and below-ground interactions, where organisms, such as symbiotic mycorrhizal fungi and insect herbivores, indirectly influence each other (Bezemer and Van Dam, 2005; Gilbert and Johnson, 2015). In this study, potato roots were inoculated with three levels of *G. intraradices* spore densities to achieve distinct levels of root colonization before adding cabbage looper larvae to plants. We showed that larvae that fed on mycorrhizal plants with low levels of colonization gained significantly less biomass compared to larvae that fed on highly colonized plants. We also found that high levels of colonization by *G. intraradices* increased shoot biomass. Finally, defense-related genes were slightly induced in insect-damaged mycorrhizal plants, but their levels of expression were not significantly different between treatments. This study suggests that AM fungus colonization affected potato 'resistance' towards a leaf-chewing insect in a 'dose-dependent' fashion.

Highly colonized mycorrhizal plants caused leaf-chewing insects to gain significantly more mass than those that fed on plants with low levels of *G. intraradices* root colonization. However, feeding on mycorrhizal plants, regardless of colonization

level did not cause *T. ni* larvae to accumulate less mass as we had originally hypothesized. Feeding on highly colonized plants resulted in heavier insects after the feeding period (Fig. 3). This could be attributed to increased shoot biomass accumulation in these plants. Mycorrhizae mediate several traits in their hosts, including water content and nutritional status (Goverde et al., 2000; Bárzana et al., 2012). The increased shoot biomass accumulation in the highly colonized plants (Fig. 1) could have contributed to an increase in larval weight. Previous research examining well-established mycorrhizal symbioses on caterpillar weight has shown similar results. Generalist caterpillars gained more mass after feeding on highly colonized (69% on average) *Plantago major* plants inoculated with *Rhizophagus irregularis* (Tomczak et al., 2016). Although not significant (P=0.0682), in this experiment, insects gained noticeably less mass when feeding on less colonized plants compared to non-mycorrhizal plants. There was no difference in the above-ground dry biomass between the non-mycorrhizal and low colonization treatments (Fig. 1A).

Both dry shoots and roots of potato plants showed no differences between non-mycorrhizal and mycorrhizal treatments, except at the high level of colonization (Fig. 2). Shoot fresh weights of mycorrhizal potatoes were only significantly increased in the highly colonized plants (Fig. 1A). Previous work has shown that *G. intraradices* colonization contributes to increased potato shoot weight in both laboratory and field trials (Hijri, 2015; Parvizi and Dashti, 2015). Marginal differences between low and medium shoot fresh weights could have been attributed to different levels of water in the plants, as the discrepancy was eliminated when shoots were dried (Fig. 1B). While it has

been established that mycorrhizae can alter root mass and architecture (Gamalero et al., 2004; Wang et al., 2011), root biomass was similar in all treatments.

The activation of defensive responses in plants depends on the feeding method utilized by the feeding insect (De Vos et al., 2005). Generalist chewing insects tend to be negatively affected by mycorrhiza (Koricheva et al., 2009). This difference is thought to be caused by generalist insects being more sensitive to plant toxins, which specialists have evolved to overcome through their selective diet (Ali and Agrawal, 2012). Previous studies with tomato have shown that mycorrhiza contributed to increased transcription of defense-related genes and ultimately discouraged herbivory (Song et al., 2013). However, this is not always the case, as foliar-feeding insects have performed better on mycorrhizal plants (Tomczak et al., 2016). In this experiment, while insightful, AM symbiosis did not significantly influence insect herbivory. However, several defense-related genes were slightly induced in all mycorrhizal plants. It is possible that despite not finding differences in gene expression among mycorrhizal plants, insects gained the most weight on highly colonized plants because of their increased size and nutritional status (Fig. 1A) (Tomczak et al., 2016). Studies have shown that an increase in JA levels can negatively affect insect herbivory (Baldwin, 1998; Moore et al., 2003; Schmelz et al., 2003; Scott et al., 2010). The results here suggest that low levels of *G. intraradices* colonization negatively affected cabbage looper's larval weight, and that mycorrhiza-induced shoot growth of potato was only detected at high levels of *G. intraradices* colonization.

CHAPTER III

ARBUSCULAR MYCORRHIZAL SYMBIOSIS INDUCES DEFENSE GENES IN POTATOSHOOTS AFTER HERBIVORY BY A CHEWING INSECT

Abstract

Throughout ecosystems, arbuscular mycorrhizal fungi (AMF) form relationships with the roots of plants, altering their hosts' nutritional status through increased uptake of phosphorus and nitrogen. This feature of AMF influences the plant's ability to overcome stresses in the environment and can influence plant-insect herbivore relationships. In this study, potato plants (*Solanum tuberosum*) were either inoculated with the AM fungus *Glomus intraradices* or mock inoculated, and plants were exposed to herbivory by cabbage looper (*Trichoplusia ni*) larvae for five and eight days. Our goal was to evaluate both the bottom-up (AMF to insect) as well as top-down (insect to AMF) interactions. Gene expression analyses were carried out focusing on phytohormone biosynthesis genes and defense genes. Our results revealed that the low levels of AM fungus root colonization used in this experiment did not change potato shoot biomass. Larvae gained more weight when they fed on non-mycorrhizal plants compared to mycorrhizal plants. We found that genes involved in activating plant defenses in shoots were induced in insect-damaged plants (± *G. intraradices*). The data indicate that mycorrhizal plants are better 'prepared' to defend themselves against cabbage loopers compared to non-mycorrhizal plants.

Introduction

The majority of terrestrial plant species form root symbiosis with arbuscular mycorrhizal fungi (AMF) found in soil (Brundrett, 2009). AMF facilitate the transfer of nutrients such as phosphate and water to the host plant in exchange for carbohydrates (Allen, 2011; Smith et al., 2011). In addition to assisting with nutrient uptake (Ortas, 2012), mycorrhizal associations improve the surrounding soil structure (Rillig et al., 2015) and play important roles in nutrient cycling (Hodge et al., 2001), ecosystem biodiversity, and productivity (Van Der Heijden et al., 1998). Mycorrhizal plants can benefit from an improved nutrient uptake and are able to allocate more resources in the formation of reproductive organs (Gange and Smith, 2005). In turn, more pollinators are attracted to mycorrhizal plants. This is one of the ways that AMF can indirectly influence and alter the behavior of organisms through direct connection to their plant hosts.

AMF are integral to plant growth and aid in acquiring macronutrients, influencing physiology, metabolism, and phytohormone balance (Hause and Fester, 2005; Parniske, 2008). Phytohormones are signaling molecules that mediate plant responses to biotic and abiotic stress (Pieterse et al., 2009). A handful of these phytohormones, namely salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), and ethylene (ET), are known to alter the AM symbiosis. Plants overproducing ET and SA showed reduced or no colonization by AMF (Medina et al., 2003; Fracetto et al., 2013). Mechanical wounding in *Medicago truncatula* leaves was shown to increase JA levels and colonization by the AM fungus *Glomus intraradices* (Landgraf et al., 2012). AM fungus colonization in tomato (*Solanum lycopersicum*) was three times higher in mutants insensitive to JA (Herrera-Medina et al., 2008). Elevated levels of JA and ABA have also been detected in roots colonized by

Glomus species (Bothe et al., 1994; Hause et al., 2002). When tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) were inoculated with the same AM fungal species, tobacco had lower SA levels (Medina et al., 2003), while SA levels increased in rice (Blilou et al., 2000). Mycorrhizal plants often exhibit enhanced resistance to stress, thought to be resulting from altered phytohormone homeostasis (Fernández et al., 2014). The outcomes of mycorrhizal associations with their hosts function in a species-specific manner (López-Ráez et al., 2010). Many potential combinations of plant-AMF relationships have yet to be explored, as the outcomes of these relationships vary depending on the species of plant and fungus involved (López-Ráez et al., 2010; Vannette and Hunter, 2013).

Plant defenses against insects are regulated through phytohormone signaling pathways (Pieterse et al., 2009). Different insect feeding guilds trigger distinct gene expression profiles of SA, JA, and ET biosynthetic and signaling pathways within the plant (De Vos et al., 2005). Phloem feeders cause minimal damage to plants and trigger responses different from leaf-chewing insects (Moran and Thompson, 2001; Schmelz et al., 2003). Feeding by lepidopteran larvae increases gene expression of the gene allene oxide synthase 1 (*AOS1*) which encodes an enzyme whose expression is directly linked to early biosynthesis and regulation of JA (Maucher et al., 2000; Ziegler et al., 2001). Levels of JA within the plant have shown to have a strong correlation to the plant's ability to deter herbivory from the phytophagous tobacco hornworm (*Manduca sexta*) in tomato (Chen et al., 2005). In response to AMF colonization, JA-related defenses have been hypothesized to enter a 'primed' state, increasing the speed and strength of response to future attack by insect herbivores (Jung et al., 2012).

Meta-analyses of the current research involving three-way interactions between leaf-chewing insects, plants, and AMF point towards polyphagous insects being negatively affected by AMF (Koricheva et al., 2009). However, AMF do not negatively affect insects in all instances. In bittersweet nightshade (*S. dulcamara*) plants, colonization by *Rhizophagus irregularis* followed by application of JA stimulated the activity of protease inhibitor (PI) proteins to be greater than plants treated with only JA (Minton et al., 2016). Protease inhibitors production is triggered by JA and has prominent roles in plant defense against generalist insects (Koiwa et al., 1997; Hartl et al., 2010). In the case of Minton et al. (2016), AMF influenced plant defenses, but the presence of AMF did not affect herbivory by the tobacco hornworm. Conversely, in tomato inoculated with *G. mosseae*, two JA biosynthesis genes (lipoxygenase D, *LOXD* and allene oxide cyclase, *AOC*) and wound response genes (*PI-I* and *PI-II*) were significantly induced by simultaneous AM symbiosis and cotton bollworm (*Helicoverpa arimigera*) herbivory (Song et al., 2013). In this instance, larvae were negatively affected when insects fed on mycorrhizal plants, gaining 62% less weight versus those that fed on non-mycorrhizal plants. It has been shown that the AM fungus inoculum, used either as a single species or as a mixture of species, can lead to different results. Root colonization by *G. mosseae* and *G. fasiculatum* resulted in decreased survival and weight of foliar-feeding black vine weevil (*Otiorhynchus sulcatus*), but only when each AM fungus was used separate as a single inoculum (Gange, 2001). The effect of insects on AM fungus colonization is even less clear. Plants allocate carbon away from shoots to roots during insect attack (Holland et al., 1996), which could potentially benefit AMF colonization. Generally, herbivory has shown to reduce AM fungus colonization (Barto and Rillig,

2010), yet inconsistencies still exist. Feeding by weevils on peas (*Pisum sativum*) for 10 days, significantly increased AM fungus root colonization (Wamberg et al., 2003). However, after 16 days of continuous herbivory, AM fungus root colonization significantly decreased compared to root colonization levels on plants that had no herbivores. The lack of consistent results in AMF-plant-insect interactions support the need to understand how three-way interactions function.

In this experiment, we explored the multifaceted relationship between potatoes and two common organisms in their environment, the below-ground AM fungus *G. intraradices* and the generalist, leaf-chewing insect in its larval stage, the cabbage looper (*Trichoplusia ni*). Our overall goal was to examine potato plant defense gene expression during tripartite interactions between cabbage loopers, potatoes, and *G. intraradices.* The specific aims of this research were to determine whether the AM symbiosis increases potato shoot biomass, to assess cabbage looper fitness, measured as larval weight, after feeding on mycorrhizal and non-mycorrhizal potato shoots, to quantify changes in potato defense-related gene expression, and to determine whether cabbage looper herbivory on shoots affected AM fungus root colonization. We hypothesized that AM fungus root colonization would not increase potato shoot biomass, cabbage looper larvae would weigh less after feeding on mycorrhizal plants compared to those that fed on non-mycorrhizal plants, transcripts levels of defense genes would increase on insect-damaged mycorrhizal plants, and AM fungus root colonization would be unaffected by insect herbivory.

Methods

Plant Growth Conditions and Arbuscular Mycorrhizal Fungus Inoculation

In vitro propagated *Solanum tuberosum* (potato, 'Désirée') plantlets were maintained on Murashige and Skoog medium (pH 5.7), supplemented with 20 g L^{-1} sucrose and 4g L^{-1} g phytagel (Murashige and Skoog, 1962), and grown at 22°C on a 16 h: 8 h, day: night cycle for eight weeks. Once roots developed, potatoes were transplanted to pots (12 cm W x 9 cm H). The soil substrate selected for the experiment was based on our previous results (Schoenherr and Gomez, unpublished), and was composed of 9:1 mason sand: topsoil (Pioneer Sand Company, Windsor, CO, USA). Sand was thoroughly washed with deionized water prior to being autoclaved (121°C, 20 PSI, 60 min). Topsoil was sieved (USA Standard sieve no. 16 and 8) before autoclaving for three cycles (121°C, 20 PSI, 60 min). Newly transplanted potatoes were kept under plastic domes to maintain high humidity. *G. intraradices* spores provided by Dr. Maria Harrison (Boyce Thompson Institute for Plant Research) were maintained on bi-plates with *Daucus carota* roots (St-Arnaud et al., 1996). Growth medium containing *G. intraradices* was dissolved using 10 mM sodium citrate (pH 7.9) to retrieve the spores. The total number of spores was estimated after resuspension in deionized water to produce the spore inoculum. This experiment was designed based on our previous results in which mycorrhizal potatoes at a 'low' level of *G. intraradices* root colonization were subjected to cabbage looper herbivory (Schoenherr and Gomez, unpublished). A 'mock' filtered inoculum was obtained from the final rinse of the spore preparation.

After a seven-day acclimation period, plants were transplanted to new pots containing 250 mL of sterile substrate (9:1 sand: soil). Potato roots received 250 *G.*

intraradices spores. Non-mycorrhizal plants were 'mock' inoculated with liquid from the spore preparation. Sand and soil from the original pot was re-used to cover the roots. Plants were fertilized twice per week with 35 ml half-strength modified Hoagland's solution with reduced P (100 µM P) to promote AM fungus colonization (Liu et al., 2007). Plants were grown at 20-23°C under four fluorescent bulbs with a 16 h photoperiod using fluorescent bulbs (113-148 µmol $m^{-2}s^{-1}$ lighting). Colonization levels were evaluated using extra plants that were inoculated to gauge mycorrhizal progression. Roots were cleared in 10% (w/v) KOH for 6 h at 85°C, rinsed with deionized water, and stained with 5% (v/v) Schaeffer black prepared in 5% (v/v) acetic acid (Vierheilig and Piché, 1998). *G. intraradices* root colonization was quantified using the gridline-intersect method (Mcgonigle et al., 1990). Extra plants that were inoculated showed colonization levels of 10% after 39 days post-inoculation (dpi). At 54 dpi, plants were moved to the greenhouse (19-24°C day, 17-23°C night; 16 h: 8 h day: night cycle) and were grouped by treatment into BugDorm 2 insect rearing tents (BioQuip Products, Rancho Dominguez, CA, USA).

Cabbage Looper Herbivory and Sample Collection

Once potatoes acclimated to the greenhouse for 12 days, five second-instar cabbage looper (*Trichoplusia ni*; CL) larvae purchased from Frontier Agricultural Sciences, Newark, DE, USA were weighed and placed on half of mycorrhizal and non-mycorrhizal plants. Larvae fed on plants for five and eight days before being removed and immediately weighed to obtain post-feeding weight. There were seven and eight biological replicates (each plant represents a replicate) in the five- and eight-day time points, respectively. Following insect removal, shoots were separated from roots and

were weighed. A randomized subsample from each root was collected for staining to quantify colonization by *G. intraradices.* The remaining root and shoot tissues were frozen in liquid nitrogen before being stored at -80°C.

Ribonucleic acid (RNA) isolation and Complimentary deoxyribonucleic acid (cDNA) synthesis

Potato shoot and root tissues from five biological replicates were ground in liquid nitrogen using a mortar and pestle. RNA was extracted from 50 mg of tissue using TRIzol® reagent (Thermo Fisher Scientific) with minor modifications to the manufacturer's instructions. Briefly, 33.3 µl of 10% (w/v) polyvinylpyrrolidone (wt 40 000) was added following the TRIzol® step to reduce polysaccharide contamination. RNA samples (87 µl) were treated with 3 µl Turbo™ DNase (2 units μl^{-1}) (Thermo Fisher Scientific) and 10 µl of reaction buffer prior to incubation for 40 min at 37°C. Samples were further purified using chloroform and subsequent isopropanol precipitation. For cDNA synthesis, one µg of total RNA resuspended in 11 µl of RNAse-free water was mixed with 1 µl each of dNTPs (10 mM) and anchored oligo dT_{22} (500 ng μl^{-1}). The final volume of 13 µl was incubated in a T100™ thermal cycler (Bio-Rad) for 5 min at 65°C, and placed on ice. Following, 4 µl of SSIV buffer, 1 µl of DTT (100 mM), 0.3 µl SuperScript® IV (Thermo Fisher Scientific), 1.2 µl DEPC-treated water, and 0.5 µl RNase OUT were added to obtain 20 µl reactions. Samples were incubated for 10 min at 50°C followed by 10 min at 80°C. The quality of cDNA samples was evaluated by semi-quantitative PCR using the reference gene elongation factor 1-alpha (*EF1-α*). Samples were ran for 95°C for 2 min, followed by 27 cycles of 95°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, and a final 5 min at 72°C. Products were visualized on 0.5x TAE

2% (w/v) agarose gels. The three most uniform cDNAs based on band intensity from each treatment were selected for qPCR.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Potato sequences were obtained via the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) and SPUD database (potato.plantbiology.msu.edu/). Query sequences either from tomato, tobacco, *Arabidopsis*, or *Medicago truncatula* (based on gene availability) were used for BLAST searches. Once the correct potato sequences were confirmed, oligonucleotides were designed using primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) or primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Oligonucleotide sequences for target genes and reference genes are listed in Table 3. Each qPCR reaction consisted of 1 µl of cDNA template (1:3), 2 µl autoclaved MilliQ® water, 5 µl of Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µl 3 µM each of forward and reverse primers. Each 384-well plate was run on a C1000® Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The thermal profile consisted of an incubation for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and annealing/extension for 1 min for 55-60°C, ending with melt curve analysis (65-95°C incrementally increasing by 5°C). The relative expression of target genes was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Each target gene was normalized to the geometric mean of two reference genes (ΔCq). Calculations were performed as follows: Relative Expression/Fold Change = $2^{-\Delta\Delta Cq}$, where: ΔCq1 = Cq (Target gene in treated sample) – Cq (Reference gene in treated sample), ΔCq2 = Cq (Target gene in "–AMF" sample) – Cq (Reference gene in "–AMF" sample), and ΔΔCq = ΔCq1 – ΔCq2. Fold change

reduction in gene expression was obtained by taking the negative inverse of $2^{-\Delta\Delta Cq}$ (Schmittgen and Livak, 2008). Heatmaps were generated using BAR HeatMapper Plus Tool (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

Statistical Analyses

Raw data were tested for normality using the Shapiro-Wilk test. Shoot weight, root weight, and relative gene expression was analyzed using one-factor analyses of variance (ANOVA), with subsequent post-hoc comparisons using Tukey's honestly significant difference test ($P<0.05$). Student's *t* tests were performed for CL weight data. All statistical analyses were conducted using SAS (9.4 for Windows) software and Microsoft Excel.

Table 3. Oligonucleotide sequences for potato genes

Gene	Sequence (5' to 3')	Amplicon length (bp)	Plant species used to find potato sequence	Potato sequence ID
Elongation factor 1 α (*EF1-α*)	F-GAGACCTTTGCTGAATACCCAC R-TCACTTTGGCACCAGTTGG	118	Potato	Sotub06g010760.1.1
β-tubulin (β*tub*)	F-ACCAGGATGCTACAGGAGATG R-GGCAGAAATTGAACAAACCAA	119	Potato	PGSC0003DMT400025739
Allene oxide synthase 1 (*AOS1*)	F- AAATGGGTCGGAAACAGAGAACC R- GGAGGAACAATTCGACCAACAA	104	Tomato	PGSC0003DMT400043495
Allene oxide cyclase 1 (*AOC1*)	F- AGTTGTTGTGTACGGCGGTT R- GCACATCAACACCCCCACTT	119	Tomato	PGSC0003DMT400033027
12-Oxophytodienoate reducatase 3 (*OPR3*)	F- TTCACAAAGGAGCAAGTAGAGGA R- GAGATGCACGACCAACATGCC	103	Tomato	PGSC0003DMT400079356
Phenylalanine ammonia lyase (*PAL*)	F- CCTAGTAGACCACGCCTTGC R- GGGTTTCCACTTTCCAACGC	150	Medicago	PGSC0003DMT400080548
Protease inhibitor I (*PI-I*)	F- CGTTGTAATCGAGTTCGTCTTGT R- TGACATGTGGCTGCTTACTTCA	103	Tomato	PGSC0003DMT400031525
Protease inhibitor II (*PI-II*)	F- AATTGTTGTACCGCAGGAGAGG R- CCAACTTGGTTATGCTGTACTGG	99	Tomato	PGSC0003DMT400039544
9-cis-epoxycarotenoid dioxygenase (*NCED*)	F- GCCGTTCAATTCAAAAATGG R- ATTCACCAATGGCTTTAGGG	115	Tomato	PGSC0003DMT400071048

Results

Potato Shoot Biomass Decreased After Eight Days of Feeding by Cabbage Looper Larvae

Potato shoot growth was not significantly affected by *G. intraradices* root colonization or by five days of cabbage looper herbivory (Fig. 5A). While cabbage loopers did noticeably lower the above-ground portions of all plants they fed on, it was not statistically significant. After eight days of continuous feeding, the shoot weights of

all plants exposed to *T. ni* larvae was significantly decreased (Fig. 5B). Our data indicate that non-mycorrhizal plants exposed to herbivory for eight days had less shoot mass compared to both treatments that were free from insects.

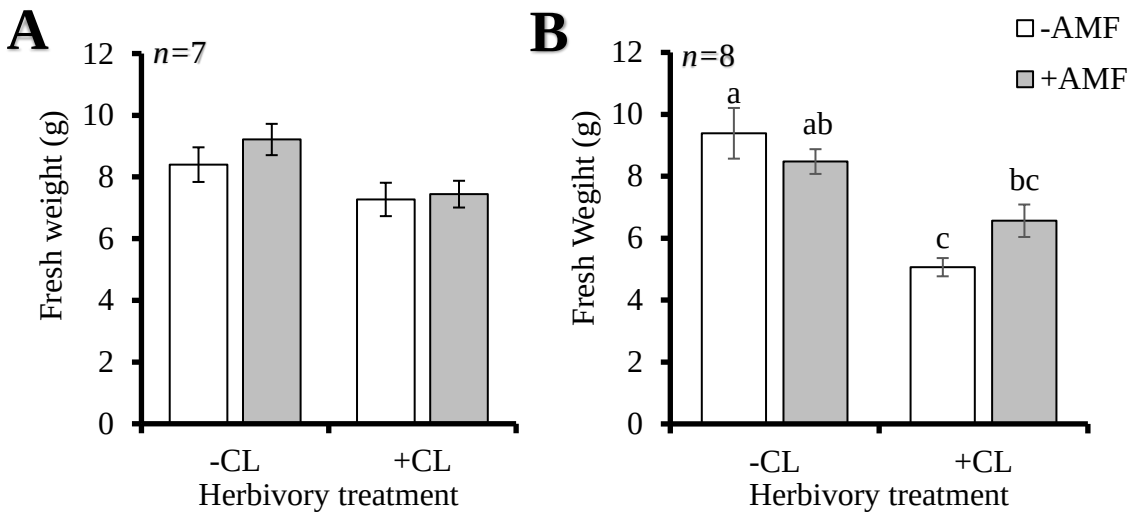

Figure 5. Effect of *Glomus intraradices* root colonization and cabbage looper (*Trichoplusia ni)* herbivory on aerial portions of potato (*Solanum tuberosum*) plants. Weight of shoots after five days (A) and eight days (B) of cabbage looper herbivory. Cabbage looper larvae were added to plants once the target AM fungus root colonization level was reached. Values represent mean ± SE. Biological replicates for each treatment indicated along y-axis. Different letters indicate significant differences based on Tukey's HSD ($P<0.05$). Open bars represent non-mycorrhizal, plants (-AMF), shaded bars represent *G. intraradices*-colonized plants (+AMF).

Cabbage Looper Herbivory and Arbuscular Mycorrhizal Symbiosis Do Not Affect Root Biomass, but Herbivory Increases *Glomus intraradices* Root Colonization

Root fresh weight of potato was not altered by either AM symbiosis or by five or eight days of cabbage looper herbivory (Fig. 6A and B). However, AM fungus colonization was significantly affected when plants incurred herbivore damage (Fig. 7). After five days, root colonization by *G. intraradices* increased in plants exposed to herbivory, but was non-significant statistically ($P=0.0501$). At eight days, the discrepancy

between the two groups became more noticeable and herbivory significantly increased the progression of the AM symbiosis (Fig. 6B).

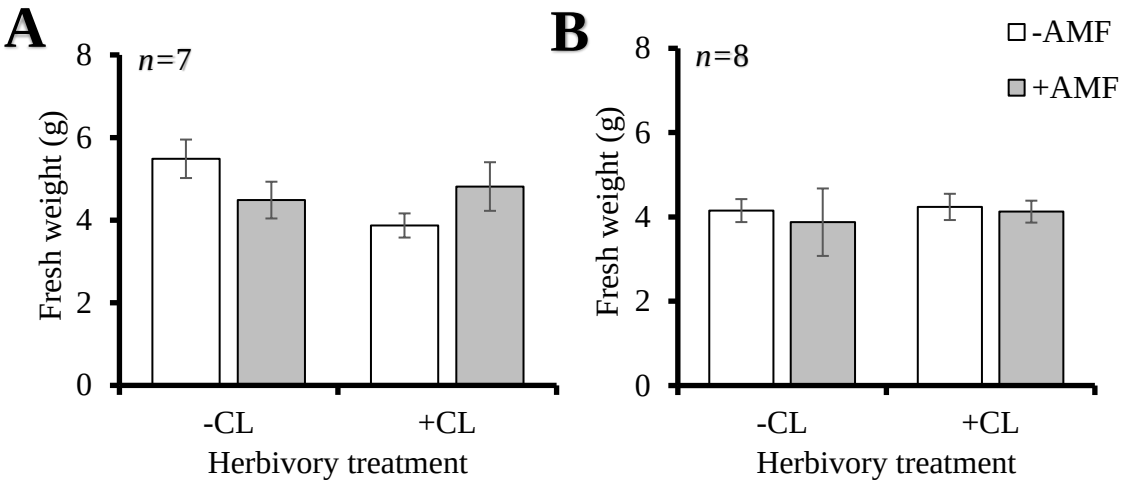

Figure 6. Impact of cabbage looper (*Trichoplusia ni)* herbivory and *Glomus intraradices* colonization on root growth of potato (*Solanum tuberosum*) plants. Fresh weight of roots after five days (A) and eight days (B) of cabbage looper herbivory. Cabbage looper larvae were added to plants once the target AM fungus root colonization level was reached. Values represent mean ± SE. Biological replicates for each treatment indicated along y-axis. Open bars represent non-mycorrhizal plants (-AMF), shaded bars represent *G. intraradices*-colonized plants (+AMF).

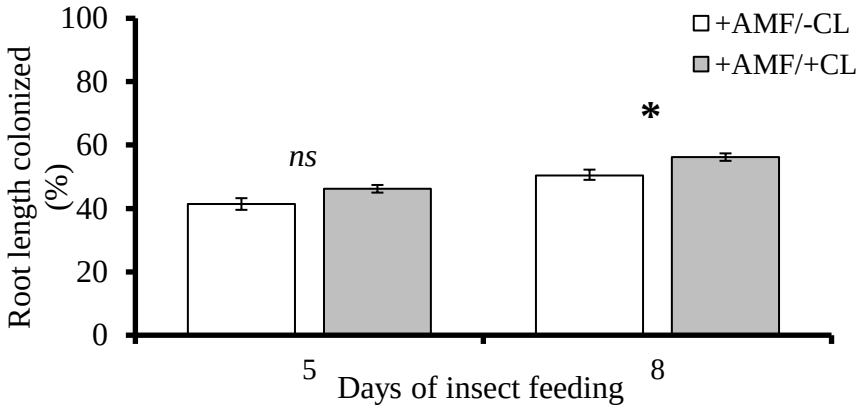

Figure 7. Percent potato (*Solanum tuberosum*) root length colonized by *Glomus intraradices* following five and eight days of cabbage looper (*Trichoplusia ni)* feeding. Five-second instar *T. ni* larvae were placed on *S. tuberosum* and fed for either five or eight days. Values represent mean ± SE. Asterisk (*) denotes significance based on two-tailed Student's *t*-test ($P<0.05$). Open bars represent herbivory-free mycorrhizal plants (-CL), shaded bars represent mycorrhizal plants exposed to cabbage looper herbivory (+CL).

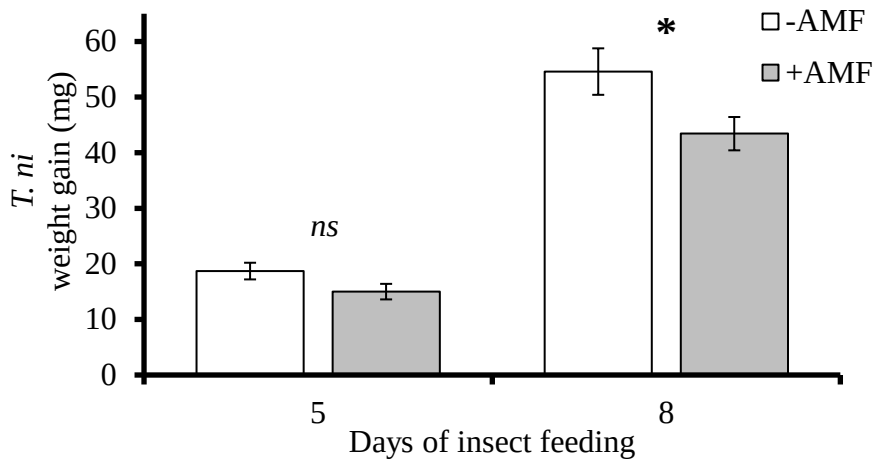

Figure 8. Changes in *Trichoplusia ni* larval weight after feeding on mycorrhizal potato (*Solanum tuberosum*) plants for five and eight days. Five second-instar cabbage looper larvae fed on mycorrhizal and non-mycorrhizal plants. *T. ni* larvae fed on potato shoots for five days or eight days and were weighed immediately after removal. Weight gain was calculated by subtracting the pre-feeding from the post-feeding fresh weights. Values represent mean ± SE. Asterisk (*) denotes significance based on two-tailed Student's *t*-test ($P<0.05$). Open bars represent non-mycorrhizal plants (-AMF), shaded bars represent mycorrhizal plants (+AMF).

Cabbage Looper Larvae Gain Less Weight After Feeding Continuously on Mycorrhizal Plants

Weighing *T. ni* larvae before and after feeding showed no significant differences between larvae that fed on mycorrhizal versus those that fed on non-mycorrhizal plants for five days (Fig. 8). While small differences existed, there were not substantial. Insects that fed for eight days on plants colonized by *G. intraradices* gained significantly less mass than insects that fed non-mycorrhizal plants ($P=0.48$), showing that colonization by *G. intraradices* had a direct effect on the growth of *T. ni* larvae during the feeding.

Figure 9. Heatmap of fold changes in shoot gene expression after five and eight days of herbivory by cabbage loopers on mycorrhizal (+AMF) and non-mycorrhizal plants (-AMF).

		5 days of feeding				8 days of feeding			
		-AMF		+AMF		-AMF		+AMF	
Pathway	**Gene**	+CL	+AMF	+CL		+CL	+AMF	+CL	
Jasmonic acid	*AOS1*	2.4	-1.4	7.9	*	38.0	-1.9	47.1	*
	AOC1	2.1	-1.0	5.6		16.9	-1.0	31.7	*
	OPR3	-1.3	-1.9	-1.4		2.5	-2.3	4.0	*
Abscisic acid	*NCED*	1.5	1.1	1.4		3.7	-1.5	3.9	
Phenylpropanoid	*PAL*	3.6	1.2	7.5	*	6.5	-1.5	12.4	*
Protease inhibitors	*PI-I*	136.9	1.3	263.4	*	41.5	-1.6	91.6	*
	PI-II	31.4	1.5	14.1	*	-1.1	-44.1	-3.0	

Fold changes in gene expression were calculated relative to the control treatment (-AMF/-CL) using elongation factor 1 alpha (*EF1a*) and β tubulin (*β tub*) as reference genes. Values represent the mean of three biological and two technical replicates per treatment. Asterisk (*) denotes significant difference among treatments for that gene determined by one-way ANOVA ($P<0.05$). Increased transcript levels for that gene relative to control (-AMF/-CL) is shown in red, and decreased transcript levels for that gene is shown in green. AMF = arbuscular mycorrhizal fungus, CL = cabbage looper.

Cabbage Looper Herbivory Induces an Increase in Transcript Levels of Defense-Related Genes in Shoots, and This Response is Enhanced in Insect-Damaged Mycorrhizal Plants

Transcript levels in shoots of the *AOS1* gene, an early gene in the JA biosynthetic pathway, decreased in the presence of only *G. intraradices*, were induced in plants exposed to five and eight days of cabbage looper herbivory, and were highly induced in insect-damaged mycorrhizal plants (Fig. 9, Appendix Table 6). After eight days of herbivory, other genes in the JA biosynthesis pathway had increased levels of transcripts (*AOC1* and *OPR3*) in insect-damaged plants, but were not statistically different from one another. At both five and eight days, *PAL*, an essential enzyme in the biosynthesis of lignin (Boerjan et al., 2003) was highly induced in insect-damaged mycorrhizal plants. Protease inhibitor I (*PI*-I) transcripts were significantly ($P<0.001$) induced by cabbage looper feeding, but the difference was even greater in mycorrhizal plants (Fig. 9). Neither

herbivory nor AM symbiosis influenced expression of any of the tested defense-related genes in roots (Fig. 10, Appendix Table 7).

Figure 10. Heatmap of fold changes in root gene expression after five and eight days of herbivory by cabbage loopers on mycorrhizal (+AMF) and non-mycorrhizal plants (-AMF)

Pathway	Gene	5 days of feeding			8 days of feeding		
		-AMF +CL	+AMF	+AMF +CL	-AMF +CL	+AMF	+AMF +CL
Jasmonic acid	*AOS1*	4.0	4.3	4.6	2.5	2.3	4.8
	AOC1	1.7	2.4	1.5	3.7	-1.1	1.8
	OPR3	1.3	2.1	1.9	2.2	1.8	2.2
Phenylpropanoid	*PAL*	1.5	1.1	1.4	3.7	0.7	3.9
Protease inhibitors	*PI-I*	2.0	6.9	2.4	4.3	2.3	1.6
	PI-II	2.2	5.6	2.0	-3.0	-2.6	-1.0

Fold changes in gene expression were calculated relative to the control treatment (-AMF/-CL) using elongation factor 1 alpha (*EF1a*) and β tubulin (*β tub*) as reference genes. Values represent the mean of three biological and two technical replicates per treatment. Asterisk (*) denotes significant difference among treatments for that gene determined by one-way ANOVA ($P<0.05$). Increased transcript levels for that gene relative to control (-AMF/-CL) is shown in red, and decreased transcript levels for that gene is shown in green. AMF = arbuscular mycorrhizal fungus, CL = cabbage looper.

Discussion

In general, mycorrhizal plants tend to increase shoot biomass, enhance nutritional status, and experience changes in gene expression that are part of a functional AM symbiosis (Parniske, 2008; Smith et al., 2011; Gallou et al., 2012). Potato responds well to associations with AMF, and increases in yield have been reported (Baum et al., 2015; Hijri, 2015). Given the level of colonization reached by mycorrhizal plants in this experiment, we did not detect significant changes in shoot biomass (Fig. 5). Eight days of feeding by cabbage looper larvae reduced shoot biomass of plants (Fig. 5B), which agrees with previous results (Borowicz, 2013). Insect-damaged mycorrhizal plants weighed less relative to undamaged, non-mycorrhizal plants (Fig. 5B). The data support our stated hypothesis that AM symbiosis would not increase shoot biomass because

plants were not highly colonized, as plants were approximately 40% colonized at the time of insect exposure in this experiment. This is consistent with previous meta-analyses that showed a positive relationship between increased AM fungus root colonization and plant biomass. A separate study showed that the extent of AM fungus colonization affects phenotypic responses in *Datura stramonium* plants in a curvilinear manner (Garrido et al., 2010; Treseder, 2013).

In this experiment, root biomass was not affected by either AM symbiosis or insect herbivory. A previous study showed that root growth varied when plants were colonized by two AM fungal species and were damaged by vine weevils. All plant roots, regardless of mycorrhizal inoculum, exposed to above-ground weevil herbivory had significantly less root biomass compared to non-mycorrhizal plants (Gange, 2001). Strawberry (*Fragaria x ananassa)* roots colonized by a single AM fungal species weighed significantly more than roots from non-mycorrhizal plants and those from plants colonized by multiple species of *Glomus*. In another study, six species of milkweed (*Asclepias* sp.) plants inoculated with a mycorrhizal fungal mixture in high and low amounts showed mixed responses, with root masses increasing under low levels of colonization and others decreasing (Tao et al., 2016). In terms of below-ground colonization by AMF, our results did not support the hypothesis that colonization would be unaltered. The increase in *G. intraradices* root colonization confirmed previous research where mycorrhizal peas, after being damaged for 10 days by foliar-feeding weevils showed increased AM fungus root colonization (Wamberg et al., 2003).

There was no difference in larval weight gain after five days of feeding (Fig. 8; P=0.09). Extending the feeding period out to eight days led to significant differences in

the biomass accumulated by larvae (Fig. 8). When larvae fed on non-mycorrhizal plants, they gained an average of 54.6 mg compared to 43.4 mg gained by those that fed on mycorrhizal plants. Our results agree with previous data that showed reduced insect weight after feeding on mycorrhizal plants (Koricheva et al., 2009). The AM fungus root colonization levels used in our experiment was similar to previous work in tomato, where the polyphagous larvae of cotton bollworm (*Helicoverpa armigera*) gained less biomass after feeding on tomato plants colonized by *G. mosseae* (Song et al., 2013). Additionally, the same changes in gene expression that were detected in shoots in our study were also reported in tomato damaged by *H. arimgera*. However, AM symbiosis does not always have negative effects on insect herbivores. Depending on the diversity, extent of root colonization by AMF, the specialization of the leaf-chewing insect, and the plant species, positive effects on insect herbivores have also been reported (Vannette and Hunter, 2013; Tomczak et al., 2016).

Gene expression analyses were performed to determine whether the AM symbiosis affects the expression of defense-related genes in potato damaged by cabbage looper larvae. A number of genes showed increased levels of transcripts in shoots damaged by cabbage looper larvae (±AMF) at both time points (Fig. 9). However, insect-damaged mycorrhizal plants showed significantly increased levels of defense-related transcripts (Fig. 9, Appendix Table 6), which supports the 'priming' hypothesis. In our study, increased expression of *AOS1*, *AOC1*, *OPR3, PI-I,* and *PI-II* genes could explain the altered phenotype seen in cabbage looper larvae that fed on mycorrhizal plants as previously reported on non-mycorrhizal plants (Koiwa et al., 1997; Baldwin, 1998; Maucher et al., 2000). *PAL,* a gene encoding an enzyme responsible for lignin

biosynthesis was also greatly induced in potato shoots (Caño-Delgado et al., 2003). The modulation of plant phytohormones and secondary metabolites reported on mycorrhizal plants (Hause et al., 2002; Hause and Fester, 2005; Walker et al., 2012) could have contributed to the enhanced tolerance to insect herbivory in mycorrhizal plants.

The results from this study indicate that the AM symbiosis induces genes in the JA pathway, which is essential for responding to insects as previously reported on non-mycorrhizal plants (Baldwin, 1998; Ziegler et al., 2001). The potential increase in JA biosynthesis following insect herbivory could have contributed to the increased colonization of *S. tuberosum* roots by *G. intraradices*, as higher JA levels are known to increase AMF colonization (Wasternack, 2014). Transcriptional changes resulting from the AM symbiosis as expected were not system-wide, as potato roots showed no differences in gene expression (Fig. 10). In this study, we examined genes in the 13-LOX branch of the oxylipin biosynthesis pathway that were previously reported on mycorrhizal tomato roots (López-Ráez et al., 2010). Previous research suppressing genes early in the 9-LOX pathway in *S. tuberosum* showed an increase in AMF colonization (Morcillo et al., 2016), and could be worth examining in future three-way AMF-plant-insect systems.

In summary, the low level of *G. intraradices* root colonization used in this study did not result in increased shoot biomass in potato. Cabbage looper larvae gained less weight after feeding on mycorrhizal plants for eight days compared to non-mycorrhizal plants (Fig. 8). This negative effect on larva fitness was further supported by significant changes in JA-pathway gene expression in potato shoots modulated by the AM symbiosis. With respect to top-down effects, eight days of herbivory were sufficient to

stimulate *G. intraradices* root colonization. Based on these results, mycorrhizal potatoes exhibit mycorrhiza-induced resistance against cabbage looper larvae early in the AM symbiosis. This likely developed as a survival mechanism for both species, as mycorrhizal fungi are obligate symbionts, reliant on forming mutually beneficial relationships with plants, such as potatoes. In return, the AMF access nutrients for their hosts and seem to aid them in overcoming biotic stress caused by insect herbivores.

CHAPTER IV

SUBTLE CHANGES IN PLANT PHYSIOLOGY OCCUR IN POTATO DURING SYMBIOSIS WITH ARBUSCULAR MYCORRHIZAL FUNGI AND CABBAGE LOOPER HERBIVORY

Abstract

Arbuscular mycorrhizal fungi (AMF) form symbioses with the roots of plants, aid in the uptake of phosphate and other nutrients from the soil, and in some instances, increase the plant host ability to overcome stress such as drought, salinity, and insect herbivory. However, there is little information about physiological changes occurring in insect-damaged mycorrhizal plants. In this study, mycorrhizal and non-mycorrhizal potato (*Solanum tuberosum*) plants were subjected to herbivory by cabbage looper (*Trichoplusia ni*) larvae for five and eight days. The goal of this experiment was to examine whether changes in plant physiology induced by the symbiosis with the AM fungus *Glomus intraradices* led to mycorrhiza-induced resistance against *T. ni*. Our results revealed that *T. ni* larvae that fed on mycorrhizal plants gained significantly less mass than those that fed on non-mycorrhizal plants. In terms of plant physiology, no differences in leaf water potential were detected regardless of mycorrhizal status or insect herbivory. However, undamaged mycorrhizal plants had more shoot mass compared to non-mycorrhizal plants and insect-damaged plants (±AMF) at the eight-day time point. Undamaged non-mycorrhizal plants had the highest relative chlorophyll content, but was only significantly different from mycorrhizal, insect-free L_4 leaves at the five-day time

point. At the same time point, photosynthesis was increased in insect-free mycorrhizal plants. Ultimately, while the mycorrhiza reduced cabbage looper fitness, it did not greatly alter potato physiology during insect herbivory.

Introduction

The sessile nature of plants forces them into constant contact with potential competitors in the environment, both above and below-ground. In the case of insect pollinators, both plants and insects benefit. However, in most cases plants are attacked by phytophagous insects. Below-ground, an overwhelming majority (>70%) of flowering plants form symbioses with arbuscular mycorrhizal fungi (AMF) (Brundrett, 2009). AMF are obligate symbionts that exchange several nutrients (primarily phosphate, nitrogen, and water) in return for photosynthetic products (Parniske, 2008; Smith et al., 2011). The ancient relationship between members of the fungal phylum Glomeromycota and plants dates back over 400 million years (Remy et al., 1994) and exists between many important agricultural crops (Ortas, 2012; Baum et al., 2015). By increasing the area of nutrient acquisition, AMF generally contribute to the plant development, size, and overall biodiversity of ecosystems (Van Der Heijden et al., 1998; Smith et al., 2000; Parniske, 2008).

Through increased nutritional status, mycorrhizal plants can tolerate and overcome environmental pressures. Studies have examined these plants and their tolerance to abiotic stress, including drought (Bárzana et al., 2012; Baslam and Goicoechea, 2012), salinity (Hajiboland et al., 2010), and temperature (Maya and Matsubara, 2013). In these studies, mycorrhizal plants displayed increased photosynthetic activity, water content, as well as increased shoot and root biomass compared to non-

mycorrhizal controls. Often, the improved characteristics of mycorrhizal plants were attributed to the fungal hyphae, which can reach smaller pores within the soil to access nutrients that plant roots cannot, enlarging the nutrient base available to the host plant. (Allen, 2011). The ability to obtain more water and ions, such as potassium, sodium, and phosphate (P), contributes to increased stomatal conductance (opening), ultimately lending to greater photosynthetic activity in plants inoculated with AMF (Augé et al., 2015).

Several studies have examined how AMF colonization alters plant tolerance to biotic stress, focusing on insects. The overall outcome of tripartite insect-plant-AM fungal interactions is dependent on the fungal species used as well as the feeding guild employed by the insect (Gange et al., 1999; Gange, 2001; Vannette and Hunter, 2013). Data indicate that phloem-feeding insects, such as aphids, show increased mass, growth rate and fecundity after feeding on mycorrhizal plants. Conversely, polyphagous chewing insects, were generally negatively affected by mycorrhizae (Koricheva et al., 2009). This same meta-analysis included studies of AMF and ectomycorrhizal fungi (ECM), another below-ground symbiont that increases N uptake of its host, finding differences in insect outcomes in the presence of both mycorrhizas. These data indicate that the effects of mycorrhiza on insects are not caused by the availability of N. Similarly, even when supplemented with additional P, mycorrhizal plants display extensive differences in gene expression, volatile organic compound (VOC) production, and tolerance to pathogens and aphids compared to non-mycorrhizal plants (Liu et al., 2003; Fritz et al., 2006; Babikova et al., 2014b). Thus, the ability of plants to endure biotic aggravation extends beyond the greater availability of nutrients provided by the AM fungus.

When investigating tripartite insect-plant-AMF interactions, studies have examined macronutrient levels, VOC profiles, protease inhibitor activity, secondary metabolite production, herbivore weight change, and plant defense gene expression (Song et al., 2014; Shrivastava et al., 2015; Minton et al., 2016; Tao et al., 2016). It is well established that insects themselves alter plant phytohormone-responsive genes when feeding (De Vos et al., 2005). Insect-driven hormonal modulation can be accomplished by protein elicitors found in oral secretions from lepidopteran larvae, in attempt to suppress defense responses from the plant (Diezel et al., 2009). Likewise, mycorrhizae modulate host phytohormone levels throughout the development and duration of the symbiosis, resulting in changes in the host secondary metabolism and preconditioning of defenses, or 'priming' (Bothe et al., 1994; Hause et al., 2002; Hause and Fester, 2005; Pozo et al., 2015). This mechanism, referred to as mycorrhiza-induced resistance (MIR), prepares the plant for future attack (Jung et al., 2012). 'Primed' plants display stronger defenses toward biotic and abiotic stress, regulated by phytohormone-controlled defense responses (Conrath et al., 2006).

In this study, we examined the physiological changes in potato (*Solanum tuberosum*; 'Desirée') plants, induced by simultaneous cabbage looper (*Trichoplusia ni*; CL) herbivory and root colonization by the AM fungus *Glomus intraradices*. Traditionally, physiological parameters including water status and photosynthesis are reported in studies examining how AMF alter plant tolerance to abiotic stresses, including temperature, salinity, drought, and other stress factors (Bárzana et al., 2012; Ruiz-Lozano et al., 2012; Maya and Matsubara, 2013). However, there is limited information about changes in plant physiology during tripartite AMF-plant-insect

relationships. Recent studies have examined physiological changes brought forth by leaf-chewing insects on mycorrhizal plants. *Plantago major* plants inoculated with the AM fungus *Rhizophagus irregularis* showed higher shoot water content and increased specific leaf area compared to non-mycorrhizal plants after damage by cabbage moth larvae (*Mamestra brassicae*) (Tomczak et al., 2016*). M. brassicae* larvae that fed on mycorrhizal plants gained more mass versus those fed the non-mycorrhizal diet. The knowledge surrounding how plant physiology is influenced by above-ground herbivores and below-ground mycorrhiza lacks and should be investigated.

RNA-seq data on the close relative of potato, tomato (*Solanum lycopersicum*), showed transcriptional changes in several genes related to 'biotic and abiotic stresses', 'photosynthesis', and 'nutrient transport' when colonized by *G. intraradices* (Cervantes-Gámez et al., 2016), in above-ground tissues. The management of AMF in soils could serve as environmentally conscious alternatives to manage insect populations (Onstad, 2013) and decrease costly fertilizer use through increased nutrient uptake. The continued use of insecticides to prevent crop loss leads to generating insecticide-resistant insect populations, which threaten production of vitally important potato crops (Alyokhin et al., 2008). The goal of this research was to 1**)** evaluate physiological changes in potatoes during mycorrhizal colonization by *G. intraradices* and herbivory by cabbage loopers and 2) test the effects of the mycorrhizal symbiosis on the insect herbivore. We hypothesized that potatoes, which exhibit enhanced shoot growth, phosphorus use efficiency, and tolerance to aboveground pathogens when forming AM symbiosis (Davies et al., 2005; Gallou et al., 2011; Senés-Guerrero et al., 2014), would experience less physiological stress (higher photosynthetic rate, water potential, fresh and dry weights) compared to

non-mycorrhizal plants. Additionally, cabbage looper larvae would gain less weight after feeding on mycorrhizal plants compared to larvae feeding on non-mycorrhizal plants.

Methods

Growth Conditions and *Glomus intraradices* Inoculation

Potato (*Solanum tuberosum,* 'Desirée') plantlets were grown in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 20 g L^{-1} sucrose, and a pH of 5.7. Potatoes were grown at 22°C for 7 weeks on a 16 h: 8 h, day: night cycle. Following root development, plants were transplanted to 250 mL pots containing 9:1 mason sand: topsoil (Pioneer Sand Company, Windsor, CO, USA). Sand was washed with deionized water before use and soil was sieved (sieve no. 8) to remove any large particulates. Sand and soil were autoclaved (121°C, 20 PSI, 60 min) once and three times, respectively, to eliminate potential microorganisms. Newly transplanted plants were kept under plastic domes to maintain high humidity. One week after acclimation, plants were inoculated by combining the substrate from the original pot with soil inoculum containing a mixed strain of *Glomus intraradices* purchased from Dr. Joseph Morton (International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi, INVAM) in a 1: 30 inoculum: original soil ratio. Non-mycorrhizal plants received a 'mock' inoculum identical to the fungal inoculum, but without *G. intraradices*. Extra biological replicates of AM plants were inoculated to serve as 'test' pots to gauge the rate of *G. intraradices* colonization. Potatoes were grown under laboratory conditions (21-23°C; 16 h:8 h day: night cycle; 113-148 µmol $m^{-2}s^{-1}$ lighting) and were fertilized with 25 mL of modified half-strength Hoagland's solution with reduced phosphate (100 µM P) twice per week (Liu et al., 2007).

Insect Herbivory

When mycorrhizal plants reached our target AM fungus colonization levels (31 days post-inoculation), potato plants were moved to the greenhouse and placed in BugDorm-2 insect tents (BioQuip, Rancho Dominguez, CA, USA). The third leaf (L_3, undamaged) counting down from the shoot apex was protected from insect herbivory by using organza drawstring bags (10.2 cm x 15.2 cm), which was used to record plant changes in an undamaged leaf. A week after acclimating to the greenhouse, five second-instar CL larvae were weighed and placed on the uppermost fully-expanded leaf of experimental plants. Following insect placement, two 'test' pots determined the progression of *G. intraradices* colonization to be approximately 39%. After five and eight days of feeding, cabbage loopers were removed from plants and immediately weighed using a microbalance (MX5, Mettler-Toledo, Columbus, OH).

Plant Physiology Measurements

Relative abundance of chlorophyll was measured in L_3 and L_4 first primary leaflets using a SPAD 502 Plus (Spectrum Technologies, Inc., Bridgend, UK). The average of three readings per leaf was reported in each biological replicate. Photosynthetic rate was determined using a LI-6400XT with the extended reach 1 cm chamber on the terminal leaflet (LI-COR Biosciences, Lincoln, NE, USA). L_3 and L_4 leaves were excised at base of the petiole using a razor blade and all leaflets were removed. Petioles were placed into a 3005-series plant water status console (Soilmoisture Equipment Corp, Santa Barbara, CA, USA) to obtain plant water potential.

Plant Biomass and *Glomus intraradices* Root Colonization

Shoot and roots were weighed separately to obtain fresh weights. A random root subsample was stored in 50% (v/v) ethanol prior to clearing and fungal staining. Plant tissues were stored in paper bags and dried for 48 h at 85ºC to obtain dry weights. Root subsamples were cleared with 10% (w/v) KOH for 6 h at 85ºC and stained using 5% (v/v) Schaeffer black ink in 5% (v/v) acetic acid (Vierheilig and Piché, 1998). Stained root portions were counted and colonization was quantified using the gridline intersect method (Mcgonigle et al., 1990).

Statistical Analysis

All data were accessed for normality using the Shapiro-Wilk test. To determine if a plant's mycorrhizal status or exposure to insect herbivory effected the physiological parameters of the four treatment groups, one-way analysis of variance (ANOVA) was performed. If ANOVA indicated significance ($P<0.05$), data were subjected to post hoc analysis using Tukey's honestly significant difference (HSD) test. Differences in CL mass, AMF colonization, and L_4 leaf photosynthesis between the M/M and AMF/M treatments were analyzed using *t*-tests ($\alpha<0.05$). All statistics were performed either using SAS 9.4 for Windows or Microsoft Excel.

Results

Mycorrhiza Increases Shoot Growth in Undamaged Potato Plants

One of the most well-established features of mycorrhiza is increasing the above-ground biomass of the plants they colonize (Parniske, 2008). In this experiment, shoots and roots were unaltered by mycorrhiza or the presence of *T. ni* larvae after five days of

continuous herbivory (Table 4). No differences were detected between either dry nor fresh weights in the five-day time point. At the eight-day time point, shoots of undamaged, mycorrhizal plants weighed significantly more than all other treatments. However, this difference became less pronounced when samples were dried, as the only treatment group differing from mycorrhizal, insect-free plants shoots were the non-mycorrhizal plants fed on by *T. ni*. *G. intraradices* colonization was not affected by either presence or absence of *T. ni* herbivory at either time point.

Table 4. Potato (*Solanum tuberosum*) shoot and root growth after five and eight days of *Trichoplusia ni* herbivory.

	5D feeding					8D Feeding				
Parameter	-AMF		+AMF		P-value	-AMF		+AMF		P-value
	-CL	+CL	-CL	+CL		-CL	+CL	-CL	+CL	
Shoot fresh weight (g)	5.2	4.8	5.7	4.9	0.280	4.3b	3.8b	5.5a	4.1b	**0.001**
Root fresh weight (g)	1.4	1.4	1.8	1.5	0.090	2.0	1.7	1.9	1.8	0.544
Shoot dry weight (g)	0.5	0.4	0.5	0.4	0.310	0.4ab	0.4b	0.5a	0.4ab	**0.036**
Root dry weight (g)	0.2	0.1	0.2	0.1	0.165	0.3	0.2	0.3	0.3	0.208
Root colonization (%)	-	-	47.4	50.5	0.131	-	-	58.0	57.6	0.935

Half of non-mycorrhizal (-AMF) and mycorrhizal (+AMF) plants were exposed herbivory by five *T. ni* larvae. Masses were measured immediately after cabbage looper removal. Values represent the average of all biological replicates (n=6). Different letters indicate statistical significance per Tukey's honestly significant difference (HSD) test ($P<0.05$).

Colonization by *Glomus intraradices* Does Not Alter Water Potential After Insect Herbivory, but Increases Photosynthetic Rate

Water potential was not significantly different in any of the treatments after either five or eight days of cabbage looper feeding (Table 5). L_4 leaves showed increased photosynthetic rates in the five-day time point, with undamaged mycorrhizal plants

significantly increasing CO_2 assimilation relative to undamaged, non-mycorrhizal plants. Interestingly, while the photosynthetic rate was slightly higher in mycorrhizal *S. tuberosum* at the eight-day time point, it was no longer significant. Photosynthesis was not measured on L_4 leaves that were exposed to cabbage loopers because of the extensive leaf damage. In general, chlorophyll indices were higher in all insect-free plants, regardless of mycorrhizal treatment, with the exception of the L_4 leaves of the five-day time point. *T. ni* herbivory significantly decreased relative chlorophyll content, with non-mycorrhizal, herbivory-free plants having the highest average in almost every reported chlorophyll index.

Table 5. Potato (*Solanum tuberosum*) inoculated with *Glomus intraradices* (+AMF) or a mock inoculum (-AMF) were exposed to five second-instar *Trichoplusia ni* larvae for either five or eight days.

	5D feeding					8D Feeding				
Parameter	-AMF		+AMF		P-value	-AMF		+AMF		P-value
	-CL	+CL	-CL	+CL		-CL	+CL	-CL	+CL	
Water potential (Ψ) L_3	-6.9	-7.2	-7.3	-8.2	0.780	-6.6	-6.4	-7.2	-7.0	0.425
Water potential (Ψ) L_4	-7.4	-6.8	-7.7	-7.1	0.440	-6.5	-7.3	-7.8	-7.6	0.721
Photosynthesis (μmol CO_2 m^{-2} s^{-1}) L_3	2.4	2.1	2.6	2.0	0.170	2.2	2.8	2.1	2.5	0.513
Photosynthesis (μmol CO_2 m^{-2} s^{-1}) L_4	1.9b	-	2.2a	-	**0.042**	2.0	-	2.2	-	0.549
Chlorophyll content (SPAD unit) L_3	40.1a	37.0ab	38.7ab	34.8b	**0.035**	42.4a	31.3b	38.8a	36.6ab	**0.001**
Chlorophyll content (SPAD unit) L_4	40.2a	34.7ab	37.3b	35.1b	**0.002**	41.1a	24.7b	40.2a	27.6ab	**0.000**

Parameters were measured immediately following *T. ni* removal. Values represent the average of all biological replicates (n=6). The third-most expanded leaf (L_3; undamaged) was bagged to prevent damage from *T. ni*. The four-most expanded leaf (L_4) was exposed to herbivore damage in treatments that received insects. Different letters indicate significant differences between treatments using one-way ANOVA ($P<0.05$).

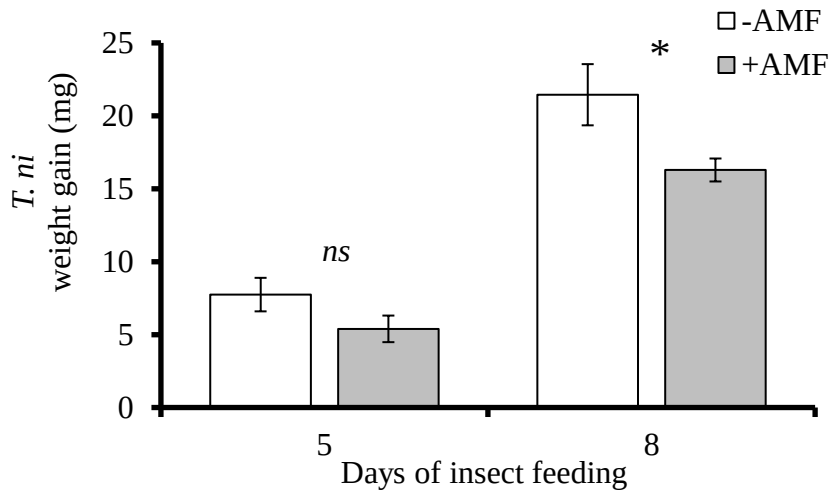

Figure 11. Effect of *Glomus intraradices* root colonization of *Solanum tuberosum* on the growth of *Trichoplusia ni*. Five second-instar cabbage looper larvae were added to each experimental plant. Mean weight gained by individual larva after feeding on potato shoots for five and eight days. Values represent mean ± standard error. Asterisk indicates significant difference based on *t*-test (α=0.05) for that time point.

Cabbage Loopers Gain More Weight After Feeding on Non-Mycorrhizal Potatoes After Eight Days

Root colonization by *G. intraradices* had an indirect impact on the growth of *T. ni* larvae after feeding on mycorrhizal plants for eight days (Fig. 1). After five days of herbivory, while small differences existed between the groups, our data were not statistically significant. The differences became more pronounced after eight days of feeding, where cabbage loopers gained significantly less mass when insects fed on mycorrhizal plants compared to those that fed on non-mycorrhizal plants.

Discussion

This work focused on examining plant physiology and plant growth parameters in mycorrhizal plants that incurred damage by a generalist leaf-chewing insect. It has been previously established that AMF influence leaf water potential and photosynthesis, and can help plants overcome abiotic stress (Sheng et al., 2008; Bárzana et al., 2012). In this

study, we found that shoot and root biomass accumulation was not nominally altered at the five-day time point, but shoot fresh weights were significantly increased in the mycorrhizal, insect-free treatment compared to all other treatments. In general, mycorrhizal plants accumulated more shoot biomass compared to their non-mycorrhizal counterparts. The difference detected at the eight-day and not five-day time point could be because the plants used in this experiment were not highly colonized at the beginning (39% at insect placement). It was shown previously that mycorrhizal plants have increased biomass when highly colonized, but this effect varies from plant to plant (Treseder, 2013). Yet this is not always the case, as seen in *Solanum lycopersicum*, where mycorrhizal plants with an average colonization of 49%, had significantly more mass compared to non-mycorrhizal plants (Hajiboland et al., 2010). Several other studies examining the impact of the AM symbiosis on plant physiology have shown a favorable trend towards the high levels of AM fungus colonization (Sheng et al., 2008; Bárzana et al., 2012; Baslam and Goicoechea, 2012; Augé et al., 2015).

When shoots are under insect attack, plants respond by allocating carbon below-ground (Holland et al., 1996). AMF rely on their host for carbon supply, so it would be expected that an increased carbon allocation to roots could potentially benefit AMF. However, in this experiment, we found that cabbage looper herbivory did not have a significant impact on *G. intraradices* root colonization (Table 4). Previous research has shown that leaf-chewing insect damage can cause an increase or decrease in root colonization by AMF (Gange, 2001; Wamberg et al., 2003). The outcomes between all members in insect-plant-AMF interactions vary significantly depending on each species involved (Borowicz, 2013; Tao et al., 2016). The same foliar-feeding insect attacking the

same plant can trigger different plant responses depending on the species of AMF colonizing roots (Gange, 2001).

The results obtained for both L_3 and L_4 leaf water potential showed no difference, regardless of treatment (Table 5). These data suggest that AM-inoculated potatoes do not display enhanced water status in the presence or absence of herbivores compared to non-mycorrhizal plants. In an earlier study, mycorrhizal *S. lycopersicum* exposed to drought stress showed increased relative water content and water potential (less negative) compared to non-mycorrhizal plants (Bárzana et al., 2012). Bárzana et al. (2012) credited the absorbent surface of the hyphae, as the AMF take up water into their hosts, as the causal factor. It has been suggested that the absorbent surface of hyphae from AMF helps with water uptake into the plant (Augé, 2001). In the experiment by Bárzana et al. (2012), all tomato plants were well colonized, with an average of 70% root-length colonized in the drought-stress plants. Moreover, results from meta-analyses indicate that highly-colonized mycorrhizal plants displayed significantly increased stomatal conductance (opening) compared to less-colonized mycorrhizal plants (Augé et al., 2015). In our experiment, after the final removal of insects, mycorrhizal plants were colonized 57% on average (Table 4). Our plants were 39% colonization at the start of the experiment, and were exposed to stress from insect herbivory. As a result, the enhanced water status was not seen. Further, all potatoes were uniformly watered throughout the experiment. Had we restricted watering, it is possible that we could have seen more substantial differences between treatments.

Because AMF help plants uptake several essential nutrients while demanding a portion of the host's carbon (Smith et al., 2011), we measured the rate of photosynthesis

as well as relative chlorophyll content in leaves that were both damaged and undamaged by *T. ni* (Table 5). Here, mycorrhizal L_4 potato leaves showed significantly higher photosynthetic rate at five days, but not at eight days post feeding. Relative chlorophyll content was different across the groups in all measured leaves at both time points. Non-mycorrhizal, insect-free plants had the highest relative chlorophyll content in L_3 leaves, but were only different from mycorrhizal plants that had been exposed to cabbage loopers. L_4 leaves showed the highest values for relative chlorophyll content in non-mycorrhizal plants free from insects, which were significantly higher than both mycorrhizal treatments, regardless of insect presence. After eight days, no difference in chlorophyll content was found between L_3 and L_4 leaves from mycorrhizal and non-mycorrhizal plants, both of which were higher than insect-exposed non-mycorrhizal plans. These results are interesting, as mycorrhizal associations are known to increase photosynthetic rates in many plants (Wright et al., 1998; Augé et al., 2015), yet this contribution is not seen here, regardless of insect exposure in the L_3 leaves. However, we did not consider total leaf area in this experiment, which is known to be impacted by mycorrhiza (Al-Karaki, 2000). Shoots in mycorrhizal, herbivory-free treatment had significantly more above-ground biomass (Table 4), likely greater leaf area, and would be photosynthesizing more. Chlorophyll content and higher SPAD readings are usually correlated with increased nitrogen content in leaves (Xiong et al., 2015). Because mycorrhiza influence nutrient uptake, including nitrogen uptake (Hodge et al., 2001), we expected higher values in mycorrhizal treatments. Had nitrogen been limited in this experiment, it is possible that we would have seen distinct phenotypic differences across treatments. Herbivory by cabbage looper negatively impacted relative chlorophyll

content, with L_4 leaves having lower values at both time points. In addition to the outright damage caused by leaf-chewing insects, oral secretions of *T. ni* could have affected the surrounding area, leading to changes in gene expression and physiology, as reported previously with lepidopteran larvae (Consales et al., 2011).

Plant tolerance to insect herbivores can be measured by examining various physiological characteristics such as plant biomass, photosynthesis, and water content. In this study, we demonstrated that mycorrhizal plants potentially exhibit antibiosis, a type of resistance that affects the biology of the pest (Fig. 1) as *T. ni* larvae gained less weight after feeding on mycorrhizal *S. tuberosum* plants for eight days. The findings here are consistent with previous research, where AM-mediated changes influence herbivory from leaf-chewing insects. Similarly, Cotton bollworm (*Helicoverpa zea*) larva fitness was altered after feeding on tomato colonized by *G. mosseae*, resulting in reduced larval weight gain (Song et al., 2013). These results were supported by examining four genes in the jasmonic acid pathway, as it plays an important role in plant defense response to chewing insects (Howe and Jander, 2008). *G. mosseae* colonization of *S. lycopersicum* was similar (~53.8% at time of harvest) to levels used in our experiment. Our previous work has also shown that 5 genes involved in plant defense responses are upregulated in insect-damaged, mycorrhizal potato (Schoenherr and Gomez, unpublished). While this work points towards low levels of colonization being effective against insects, >70% colonization by *R. irregularis* hindered beet armyworm (*Spodoptera exigua*) larval weight gain in tomato (Shrivastava et al., 2015). Cabbage moth (*Mamestra brassicae*) larvae gained more mass on mycorrhizal *Plantago major* plants (60% colonized) (Tomczak et al., 2016). Clearly, the outcomes of AMF-plant-insect interactions are

highly specific and vary depending on the plant, insect, and AM fungus used (Bennett and Bever, 2007; Koricheva et al., 2009; López-Ráez et al., 2010; Tao et al., 2016)

In conclusion, we did not find that inoculating *S. tuberosum* with *G. intraradices* significantly altered plant physiology, but did confer resistance when plants were exposed to herbivory from *T. ni*. Studies have shown that AM symbiosis grant advantages to plants when challenged by a variety of abiotic stresses (Sheng et al., 2008; Bárzana et al., 2012; Baslam and Goicoechea, 2012). However, in these experiments, plants were highly-colonized (>60% root colonization). AM fungal densities induce different phenotypic changes in their host plants and can affect how plants tolerate and respond to stress (Gange and Ayres, 1999). *Datura stramonium* plants inoculated with varied amounts of *Glomus* species had curvilinear relationships with root mass, seed production, and foliar area (Garrido et al., 2010). In this experiment, mycorrhizal plants were not highly colonized which could explain the subtle changes in plant physiology detected. However, we did see a negative impact on larval growth at this lower level of *G. intraradices* root colonization. Future experiments could address the physiological responses in potato during *T. ni* herbivory, using low and high levels of *G. intraradices* colonization to determine whether plants transition from resistance to tolerance.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In this study, we evaluated the impact of the tripartite cabbage looper-potato-AM fungus interaction on each organism involved, specifically focusing on plant physiology and gene expression. Previous work has shown that plants forming AM symbiosis have increased size, altered physiology, and are better suited to overcome biotic and abiotic stress (Wright et al., 1998; Al-Karaki, 2000; Bárzana et al., 2012). However, it has been established that the effects on each organism in the tripartite insect-plant-AMF interactions are species-specific (Vannette and Hunter, 2013; Fernández et al., 2014; Minton et al., 2016; Tao et al., 2016). Few studies focused on the effect that the extent of the AMF root colonization can have on plant-insect interactions. The plant's response to the AM fungus colonizing it seems to depend on the specific plant-fungus combinations, just as much as the progression of the AM symbiosis (Tao et al., 2016). In addition to influencing nutrient content and above-ground biomass, AM fungus abundance can also affect insect herbivore fitness (Vannette and Hunter, 2013). In the present study, we found that shoots from highly colonized potato plants had significantly more biomass compared to non-mycorrhizal plants (± insects). When potato plants were less colonized (<50% at time of insect exposure), no substantial differences in potato shoot biomass was detected, nevertheless, insect larval weight was negatively impacted.

Results from Chapter II showed that inoculation of potatoes with varied amounts of *G. intraradices* spores (low, medium, and high) had different effects on plant-insect interactions, which agrees with previous findings (Gange and Ayres, 1999; Garrido et al., 2010; Vannette and Hunter, 2013; Tomczak and Müller, 2017). Potato plants that were highly colonized had a significant increase in shoot fresh and dry weights even after seven days of cabbage looper herbivory (Fig. 1A). However, we did not detect major differences in root growth (Fig. 1B). Cabbage looper larvae gained the most mass after feeding for seven days on highly colonized plants, and gained the least mass after feeding on less colonized plants (Fig. 3). We were unable to detect significant differences in weight gain between larvae that fed on non-mycorrhizal plants and mycorrhizal plants at low, medium and high levels of AM fungus colonization (Fig. 3). However, the trend showed larvae that fed on mycorrhizal plants at low levels of AM fungus colonization gained significantly less mass compared to those feeding on highly colonized plants. There was no difference in insect weight gain between the cabbage loopers feeding on low, medium, and high levels of mycorrhizal and non-mycorrhizal plants (Fig. 3). Gene expression of four defense-related genes were tested, and mycorrhizal shoots showed a modest increase in transcripts levels compared to non-mycorrhizal plants, but the differences were not statistically significant.

We decided to use the low levels of AM fungus colonization in experiments described in Chapters III and IV. To assess plant gene expression, mycorrhizal and non-mycorrhizal plants were exposed to five and eight days of cabbage looper herbivory. Low levels of AM fungus root colonization significantly altered plants in the eight-day trial, with non-mycorrhizal shoot exposed to insects weighing significantly less than plants

free from insects, regardless of mycorrhizal status (Fig. 5B). Root biomass was not altered in any of the treatments (Fig. 6). Further, insects were negatively affected by the AM symbiosis and gained markedly less mass after eight days of feeding (Fig. 8). Opposite of our hypothesis, feeding by the cabbage looper stimulated the progression of the colonization (Fig. 7) and resulted in markedly increased *G. intraradices* root colonization at the eight-day time-point. While this increase in AM fungus colonization was unexpected, previous work has shown similar results (Wamberg et al., 2003). Defense-related genes, primarily in the JA pathway, were induced in the shoots of plants exposed to cabbage looper herbivory, but had significantly increased levels of transcripts in insect-damaged mycorrhizal plants (Fig. 9). *PAL* and *PI-I*, two genes induced by JA production with known defensive roles (Blilou et al., 2000; Song et al., 2013) were significantly induced in shoots from insect-damaged mycorrhizal plants, which could explain the altered phenotype seen in the insects. These changes were not plant-wide, as gene expression was not altered in roots (Fig. 10, Appendix Table 7).

To further investigate the tripartite relationship, we examined physiological stress responses in mycorrhizal potatoes after five and eight days of cabbage looper herbivory. The low level of *G. intraradices* colonization used in this experiment did not greatly alter plant physiology (Tables 4 and 5). At the eight-day time-point, the insect-free mycorrhizal plants accumulated significantly more above-ground biomass compared to all non-mycorrhizal and mycorrhizal plants with insects (Table 4). After drying the samples, shoot weight was different only between the mycorrhizal, insect-free plants and the non-mycorrhizal plants that had been fed upon. Water potential was not affected in damaged or undamaged leaves (Table 5). Photosynthesis was higher in undamaged leaves

from mycorrhizal plants, but only at the five-day time point. Non-mycorrhizal, insect-free plants had the highest chlorophyll content in both damaged and undamaged leaves at both time points. Similar to the previous experiment, the weight of cabbage looper larvae was significantly decreased after feeding on mycorrhizal potatoes for eight days (Fig. 11).

Future Directions

This study primarily focused on optimizing conditions to study three-way interactions between leaf-chewing insects, potatoes, and AMF. Based on the results, we found that the extent of *G. intraradices* root colonization has diverse effects on potato shoots, but also affects fitness of a chewing insect. Future three-way experiments could examine the differences in gene expression between high and low levels of AM fungus colonization. It is possible that plants transition from a 'resistance', where defense-related genes show increased transcription, to a 'tolerance' strategy once the plants are highly colonized. In this work, we demonstrated that mycorrhizal plants at low levels of *G. intraradices* colonization exhibited enhanced 'resistance' to cabbage looper herbivory, perhaps via JA-regulated defense responses. Increased nutritional status and size of highly colonized mycorrhizal plants could contribute to their ability to tolerate damage from insect herbivores. The physiological parameters that were measured in this work could be influenced at higher levels of AM fungus colonization, as colonization level is directly proportional to above-ground biomass accumulation (Treseder, 2013). The ability for a plant to tolerate insect herbivory could be closely tied to its access to nutrients and relative growth rate, which are both seen in mycorrhizal plants (Parniske, 2008).

Like this work, future studies could examine how gene expression in mycorrhizal plants changes over a period of time during insect herbivory. It is possible that measuring changes in gene expression at 0 h, 12 h, and 24 h after insect exposure could be drastically different from the results seen in the five and eight-day time points. Additionally, these studies could examine defense-related genes in other phytohormone pathways. Studies could go as far as performing RNA sequencing to obtain the full transcriptome of mycorrhizal potato plants exposed to insect herbivores at high and low levels of colonization, as several interesting changes would likely take place as the AM symbiosis progresses.

In summation, low levels of root colonization by *G. intraradices* seems to enhance the defenses of potatoes when challenged by the cabbage looper. While the work here points towards increased transcription of defense-related genes, the mechanism of mycorrhizal-induced resistance is not fully understood. We also found that low levels of *G. intraradices* root colonization of plants and insect herbivory had little impact on plant physiology given the duration of this experiment. Future research could find that these effects are greatly altered at high levels of AM fungus colonization. Investigating high versus low levels of AM fungus colonization against insects could uncover the potential 'tipping point', where mycorrhizal plants transition from enhanced resistance to strengthened tolerance.

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APPENDIX A

GENE EXPRESSION ANALYSIS

Table 6. Potato shoot gene expression after five and eight days of herbivory by cabbage loopers on mycorrhizal (+AMF) and non-mycorrhizal plants (-AMF).

		5 days of feeding				8 days of feeding			
Pathway	**Gene**	-AMF +CL	+AMF	+AMF +CL	*P*- value	-AMF -CL	+AMF	+AMF +CL	*P*- value
Jasmonic acid	*AOS1*	2.40 ±0.39b	0.70 ±1.10b	7.94 ±1.99a	0.001	38.01 ±10.89a	0.53 ±0.1b	47.05 ±6.72a	0.010
	AOC1	2.12 ±0.42	0.98 ±0.14	5.64 ±2.32	0.115	16.88 ±2.16ab	0.98 ±0.19b	31.72 ±9.24a	0.021
	OPR3	0.78 ±0.38	0.52 ±0.10	0.71 ±0.28	0.801	2.54 ±0.67ab	0.44 ±0.06b	4.02 ±0.94a	0.025
Abscisic acid	*NCED*	1.49 ±0.57	1.10 ±0.30	1.38 ±0.17	0.768	3.68 ±1.90	0.66 ±0.12	3.90 ±1.70	0.293
Phenylpropanoid	*PAL*	3.60 ±0.47ab	1.24 ±0.37b	7.47 ±1.87a	0.022	6.48 ±1.10b	0.67 ±0.25c	12.44 ±0.52a	<0.001
Protease inhibitors	*PI-I*	136.93 ±41.87b	1.25 ±0.56c	263.39 ±10.77a	<0.001	41.49 ±12.24b	0.62 ±0.46b	91.57 ±13.25a	0.002
	PI-II	31.35 ±5.65a	1.48 ±0.56b	14.10 ±5.40ab	0.009	0.92 ±0.44	0.02 ±0.01	0.28 ±0.07	0.113

Changes in gene expression were calculated relative to the control treatment (-AMF/-CL) using elongation factor 1 alpha (EF1a) and β tubulin (β tub) as reference genes. Values represent means ±SE of three biological and two technical replicates per treatment. P<0.05 denotes significant difference among treatments for that gene determined by one-way ANOVA. Different letters indicate significant difference among treatments based on Tukey's honestly significantly different (HSD) test. AMF = arbuscular mycorrhizal fungus, CL = cabbage looper.

Table 7. Potato root gene expression after five and eight days of herbivory by cabbage loopers on mycorrhizal (+AMF) and non-mycorrhizal plants (-AMF).

		5 days of feeding				8 days of feeding			
Pathway	**Gene**	-AMF +CL	+AMF	+AMF +CL	*P*- value	AMF +CL	+AMF	+AMF +CL	*P*- value
Jasmonic acid	*AOS1*	4.03 ±0.79	4.32 ±0.37	4.63 ±0.19	0.411	2.45 ±0.59	2.26 ±0.14	4.81 ±2.80	0.517
	AOC1	1.71 ±0.34	2.45 ±0.37	1.51 ±0.62	0.514	1.80 ±0.30	0.94 ±0.06	1.81 ±0.30	0.239
	OPR3	1.33 ±0.33	2.06 ±0.31	1.88 ±0.49	0.418	1.82 ±0.13	2.25 ±0.70	2.23 ±0.40	0.778
Phenylpropanoid	*PAL*	2.02 ±0.41	6.87 ±1.08	2.41 ±1.30	0.056	4.30 ±1.74	2.25 ±0.13	1.58 ±0.17	0.338
Protease inhibitors	*PI-I*	2.19 ±1.07	5.64 ±2.27	1.95 ±0.52	0.341	0.39 ±014	0.33 ±0.19	0.98 ±0.06	0.072
	PI-II	3.13 ±1.09	1.57 ±0.19	1.45 ±0.59	0.385	4.80 ±1.10	2.25 ±0.97	2.98 ±1.48	0.494

Changes in gene expression were calculated relative to the control treatment (-AMF/-CL) using elongation factor 1 alpha (*EF1a*) and β tubulin (*β tub*) as reference genes. Values represent means ± SE of three biological and two technical replicates per treatment. $P<0.05$ denotes significant difference among treatments for that gene determined by one-way ANOVA. AMF = arbuscular mycorrhizal fungus, CL = cabbage looper. AMF = arbuscular mycorrhizal fungus, CL = cabbage looper.